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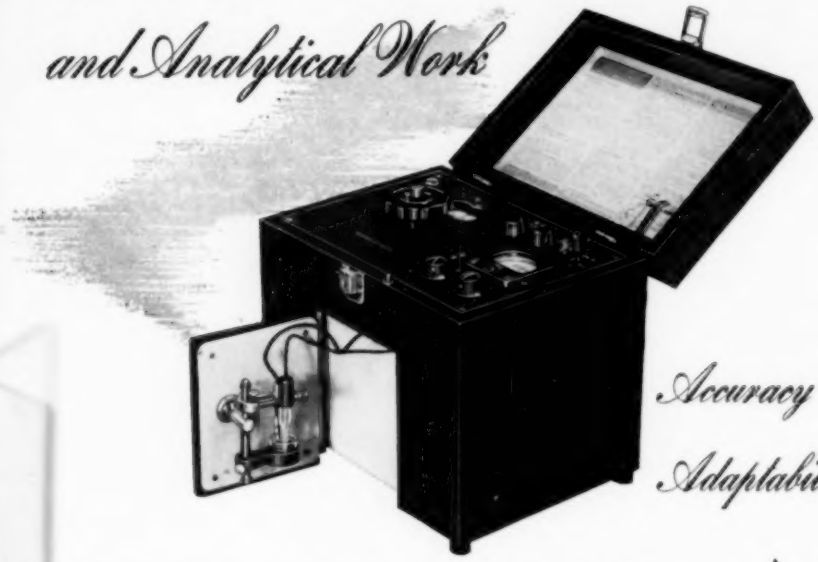

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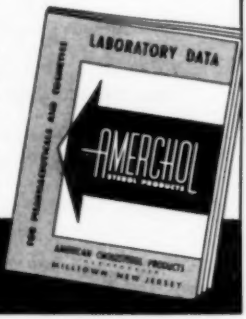

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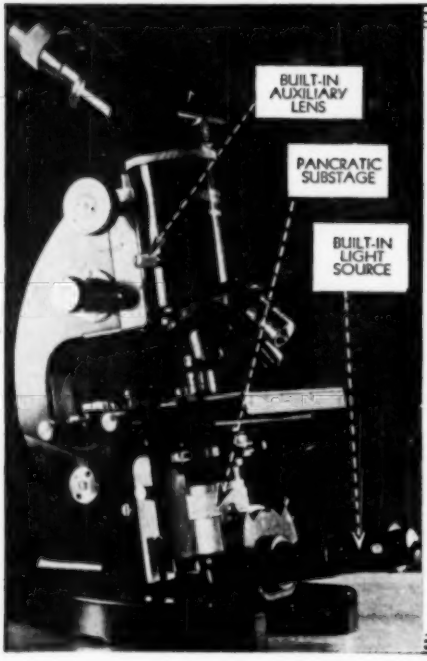

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Comparative Studies on Terramycin and Aureomycin: Antibacterial Spectrum, Serum Concentrations and Urinary Excretion*

By HENRY WELCH, FRANKLIN D. HENDRICKS, CLIFFORD W. PRICE, and WILLIAM A. RANDALL

Terramycin, a recently discovered antibiotic, is compared with aureomycin. These antibiotics are very similar in their antibacterial spectra and in the blood concentrations produced following oral doses in man. A relatively marked increase in blood concentrations occurs with an increase in dose of terramycin. This increase was not as pronounced with aureomycin. The cumulative urinary excretion and the concentration in the urine at any given time was greater for terramycin than for aureomycin.

THIS investigation is concerned with a new antibiotic, terramycin, recently described by Finlay, Hobby, *et al.* (1). Supplies of terramycin[1] were received in the form of the crystalline hydrochloride and the crystalline base. The hydrochloride was the more soluble and, since there were no apparent differences in activity between it and the free base, the hydrochloride was used in these studies. The reported (1) antibacterial spectrum of terramycin showed it to be active against a large number of bacterial species, certain rickettsiae, and large viruses. Since it is well known that wide differences exist in the resistance of various strains of bacterial species to a given antibiotic, it seemed desirable to conduct bacterial spectrum studies using strains of organisms from our own collection. A total of 54 strains of bacteria comprising 20 genera were studied. The bacterial sensitivities were determined by the double serial dilution method using penicillin assay broth as a culture medium and a 1:1000 dilution of a twenty-four hour culture of the desired organism as the inoculum. In the case of the anaerobic bacteria, fluid thioglycollate medium was used. The saprophytic acid-fast bacteria were tested in Dubos' medium. The tests were incubated at 37° for ninety-six hours and the minimal inhibitory concentration (MIC) of the antibiotic recorded for each day of the incubation period. At the end of ninety-six hours the entire contents of all tubes in the series showing no apparent growth were transferred into fluid thioglycollate medium and incubated overnight to determine the minimum lethal concentration (MLC). These data are shown in Table I.

* Received March 2, 1950, from the Federal Security Agency, Food and Drug Administration, Wash., D. C.

[1] Supplied by Chas. Pfizer and Company, who discovered and developed terramycin.

TABLE I.—BACTERIAL SPECTRUM OF TERRAMYCIN HYDROCHLORIDE

	MIC, μg./Ml. Days Incubation, 37°				MLC, μg./Ml., 4 Days
	1	2	3	4	
Clostridium botulinum	<0.05	0.2	0.2	0.4	0.80
Cl. sporogenes	0.10	0.80	0.80	0.80	6.25
Cl. tetani	0.10	0.20	0.20	0.20	0.20
Cl. perfringens	<0.025	<0.025	0.025	0.025	0.025
Salmonella paratyphi	4.0	62.5	125	250	500
S. pullorum	2.0	15.5	62.5	125	125
S. schottmuelleri	15.6	31.2	125	125	500
S. typhosa	1.0	7.8	15.6	31.2	31.2
S. typhosa resistant to *Dihydrostrep. Caprylate*	3.12	12.5	25	25	50
S. typhosa resistant to *Streptomycin Caprylate*	0.20	1.56	3.12	12.5	12.5
S. typhosa resistant to *Streptomycin Propionate*	0.20	0.80	6.2	6.2	6.2
S. typhosa resistant to *Streptomycin Undecylenate*	0.40	3.10	3.10	6.2	12.5
Aer. aerogenes	15.6	62.5	125	125	500
Esch. coli	0.10	0.78	1.56	1.56	1.56
Ps. aeruginosa	7.8	31.2	62.5	125	125
Kl. pneumonia type A	0.50	1.0	4.0	15.6	62.5
Proteus vulgaris	>500	>500	>500	>500	>500
Brucella bronchiseptica	7.8	31.2	125	250	250
Sh. dysenteriae	4.0	31.2	62.5	125	250
Sh. paradysenteriae	4.0	31.2	125	125	500
S. marcescens 1	0.40	0.78	3.12	12.5	12.5
S. marcescens 2	100	>100	>100	>100	>100
Past. pestis	<0.05	0.10	0.20	0.40	0.40
Sarcina lutea	0.25	0.50	4.0	7.8	15.6
Micrococcus pyogenes var. *aureus*	0.25	0.5	4.0	15.6	62.5
M. flavus	0.25	0.5	4.0	7.8	15.6
Bacillus subtilis 1	0.10	0.40	1.56	6.25	6.25
B. subtilis 2	7.8	31.2	62.5	62.5	62.5
B. circulans	0.25	0.5	4.0	7.8	15.6
Neis. catarrhalis	0.20	0.40	1.56	1.56	1.56
Coryn. diphtheriae	12.5	25	50	50	50
C. hofmanni	0.20	0.40	0.78	1.56	1.56
C. xerose	0.40	0.40	1.56	3.12	6.25
Streptococcus pyogenes C-203	0.40	1.56	6.25	6.25	12.5
Strep. agalactiae	<0.05	0.20	0.78	0.78	0.78
Strep. dysgalactiae	0.40	1.56	6.25	12.5	50
Strep. viridans 1	0.78	6.25	12.5	25	100
Strep. viridans 2	0.20	0.40	0.78	3.12	25
Strep. viridans 3	0.10	0.40	0.40	3.12	3.12
Strep. viridans 4	0.40	0.78	1.56	1.56	1.56
Strep. equi	<0.05	0.10	0.10	0.10	0.10
Strep. fecalis 1	0.78	1.56	6.25	12.5	50
Strep. fecalis 2	25	25	25	25	100
Strep. fecalis 3	0.78	1.56	6.25	12.5	50
Strep. fecalis 4	0.10	0.20	0.40	0.78	3.12
Strep. fecalis 5	0.78	3.12	12.5	25	100
Strep. fecalis 6	0.20	0.40	0.78	3.12	50
Vibrio comma	0.20	0.40	0.78	0.78	0.78
Torula sp.	>500	>500	>500	>500	>500
Yeast sp.	>500	>500	>500	>500	>500
Mycobacterium phlei	0.1	0.1	0.1	0.1	0.1
Myco. avium	0.1	0.1	0.1	0.25	0.25
Myco. ranae	0.1	0.1	0.1	0.25	0.25
Myco. smegmatis	0.1	0.1	0.1	0.50	0.50

It can be seen that terramycin is quite active against the clostridia and saprophytic mycobacteria and possesses a wide range of activity against the other species tested. It will be noted that this antibiotic was inactive against the yeast-like organisms and against *Proteus vulgaris*. In most cases the observed minimal inhibitory concentration increased as the incubation period was extended, and the minimal lethal concentration in many cases was much greater than that recorded from visual inspection of the tests. It is apparent that terramycin possesses largely a bacteriostatic effect rather than a bactericidal one. In this respect it is similar to aureomycin (2) which also exerts a bacteriostatic effect and has a similar wide spectrum. Differences in the bacteriostatic end points of the two antibiotics were noted on prolonged incubation of the tests and may reflect the greater stability of terramycin as compared with aureomycin in solution. Based on the twenty-four hour sensitivities, 37 of the 54 bacterial strains were sensitive to 1.0 μg. or less of terramycin, 5 strains were sensitive to from 1.0 to 5.0 μg., and 10 strains were sensitive

to more than 5.0 μg. Previous studies (2) have shown that among 53 bacterial strains covering about the same genera, aureomycin inhibited 32 strains in a concentration of 1.0 μg. or less, 10 strains in a concentration of 1.0 to 5.0 μg., and 11 strains in a concentration of more than 5.0 μg. It should be pointed out that the variation in sensitivity among strains of a given species makes it highly desirable to determine the sensitivity of the infecting organism in each case in order to evaluate properly the therapeutic usefulness of the antibiotic.

ABSORPTION AND EXCRETION OF TERRAMYCIN AND AUREOMYCIN

Preliminary studies indicate a close relationship between terramycin and aureomycin. Both drugs appear to be readily absorbed from the gastrointestinal tract and are found in the blood and urine. In addition, appreciable amounts are excreted in the feces and affect the intestinal flora. For comparative purposes parallel experiments were performed with these two antibiotics in the absorption and excretion studies.

Single doses of terramycin were administered orally to normal male subjects. Four groups of five individuals each were utilized. One group received 0.5 Gm. of terramycin hydrochloride, the second group 1.0 Gm., the third group 1.5 Gm., and a fourth group 2.0 Gm. Bloods were drawn at two, four, six, and twenty-four hours and the serum levels of terramycin determined. Urine was also collected at various intervals for the excretion studies to be described later. The same experiment was performed on other subjects using aureomycin hydrochloride.

Staphylococcus aureus (209-P) was used to determine the blood concentrations of terramycin. The test was performed in the same manner as the test for penicillin serum concentrations (3). The standard was crystalline terramycin base containing 10.0 μg./ml. and the inoculum was a 1:10,000 dilution of a twenty-four hour broth culture of the test organism. This method will detect from 0.3 to 0.6 μg. of terramycin per milliliter of serum. The method for determining aureomycin serum concentrations has been described in a previous publication (2).

For results of the average serum concentrations obtained with each dose and the individual serum concentrations for terramycin see Fig. 1.

As the doses increase there is a tendency for the serum concentrations to increase at any given time. In the case of the 1.0-Gm. and 1.5-Gm. doses it will be noted that the average serum concentration for the latter dose is lower than for the former. This may be due to patient variation; however, the increase in the average serum concentration was pronounced when doses were given in amounts differing by 100%, e.g., 1.0 and 2.0 Gm. The aureomycin serum concentrations are shown in Fig. 2.

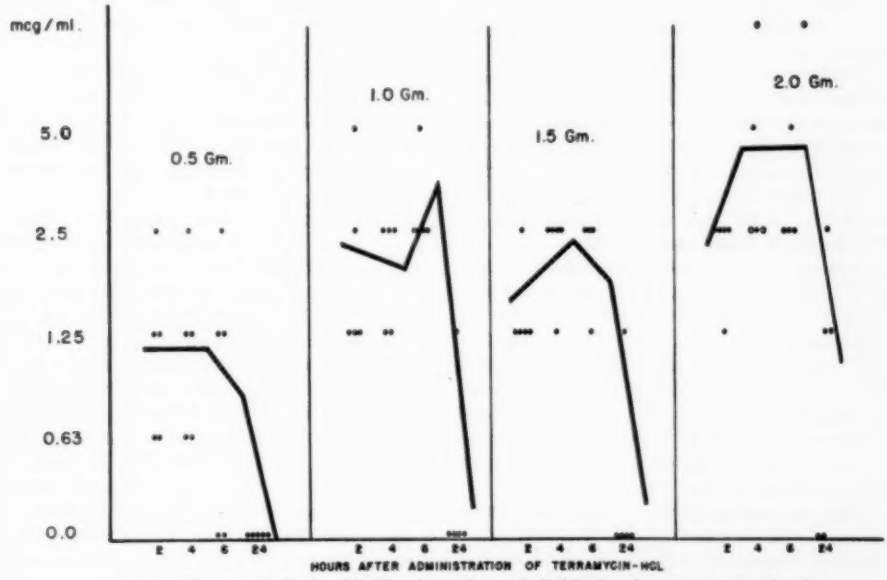

Fig. 1.—Terramycin hydrochloride, single dose: individual and average serum level.

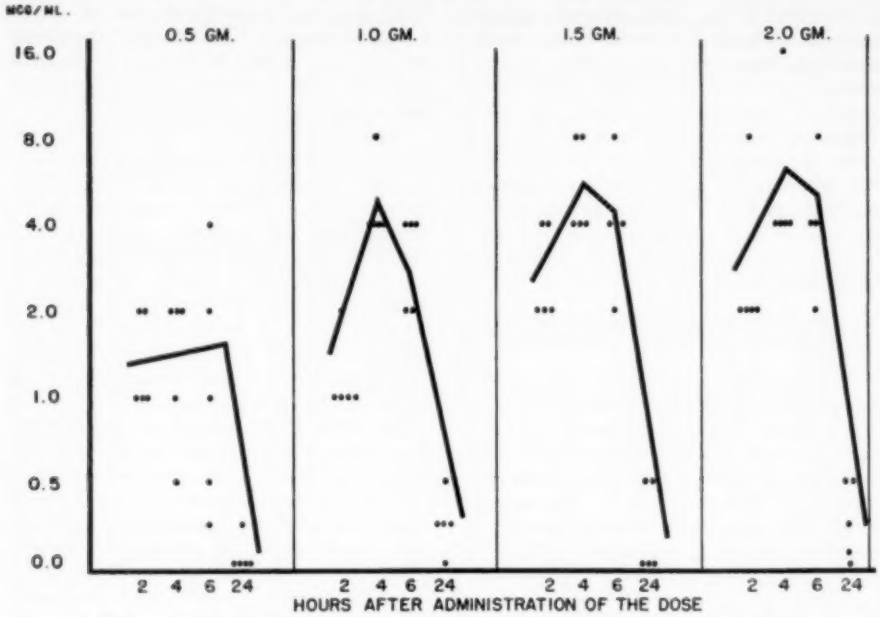

Fig. 2.—Aureomycin hydrochloride, single dose: individual and average serum level.

There is a distinct rise in the serum concentrations between the 0.5-Gm. dose and the 1.0-Gm. dose, but the 1.0-Gm., 1.5-Gm., and 2.0-Gm. doses gave essentially the same average serum concentrations. The average serum concentration obtained with aureomycin is somewhat higher than that obtained with terramycin at each dose. Both drugs in 0.5-Gm. doses gave serum concentrations of approximately 1.0 μg. for at least six hours. Toxic symptoms were not noted with either drug, although occasional patients complained of loose or soft stools.

Terramycin and aureomycin were given also in divided doses. For this study four groups of normal adult males, three individuals per group, were used. The first group received a total dose of 1.0 Gm. every six hours for a twenty-four hour period. The second group received 2.0 Gm., 0.5 Gm. every six hours; the third group 3.0 Gm., 0.75 Gm. every six hours; and the fourth group 4.0 Gm., 1.0 Gm. every six hours. The intervals at which blood was drawn, the average blood concentrations, and the individual ranges for the various groups are shown in Figs. 3 and 4 for terramycin and aureomycin, respectively.

The terramycin serum concentration rises sharply with increased dosage from 0.25 Gm. every six hours to 0.75 Gm. every six hours, and tends to level off at the 0.75-Gm. and 1.0-Gm. dose. In the case of aureomycin this rise with increased dosage is much less marked. After each dose of drug there is in general a relatively sharp increase in the serum concentration. As with single doses of these drugs some patients had soft or loose stools, although no frank diarrhea was observed.

Total urine samples were collected at two, four, six, fifteen and twenty-four hours. Assays for terramycin and aureomycin were made using a modified lutea plate technique (4). The cumulative percentage of terramycin and aureomycin excreted is shown in Fig. 5.

In the twenty-four hour period 18 to 19 per cent of the total dose of terramycin was excreted in the urine following oral doses of 0.5 Gm. and 1.0 Gm. On the larger oral doses of terramycin (1.5 and 2.0 Gm.) approximately 10 per cent of the total dose was excreted in the urine over a twenty-four hour period. In the twenty-four hour period following oral doses of 0.5 and 1.0 Gm. of aureomycin the total urinary excretion was only 8 to 9 per cent of the total dose administered and on the larger doses (1.5 and 2.0 Gm.) only 5 to 6 per cent was recovered in the urine. As was the case with terramycin, a higher percentage of aureomycin was excreted on the lower doses. On comparable doses the total amount of terramycin excreted is considerably greater than

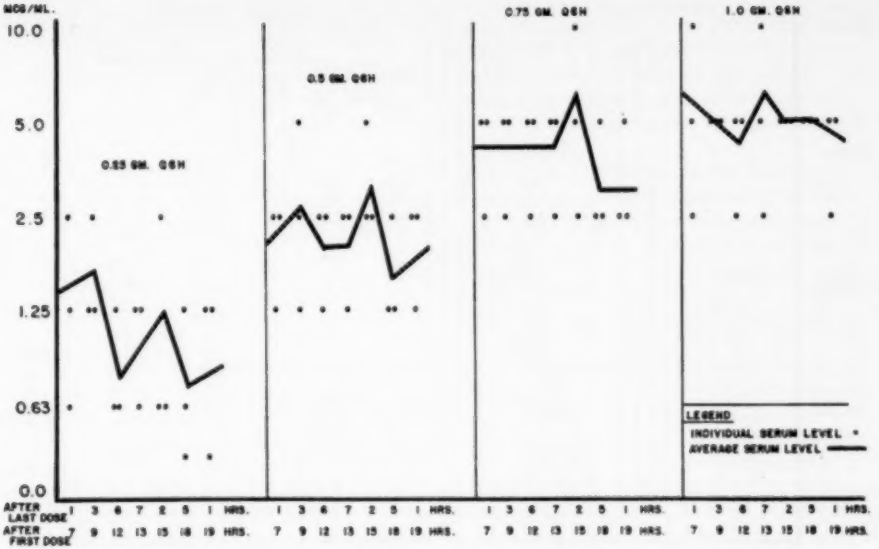

Fig. 3.—Terramycin hydrochloride in divided doses: individual and average serum levels.

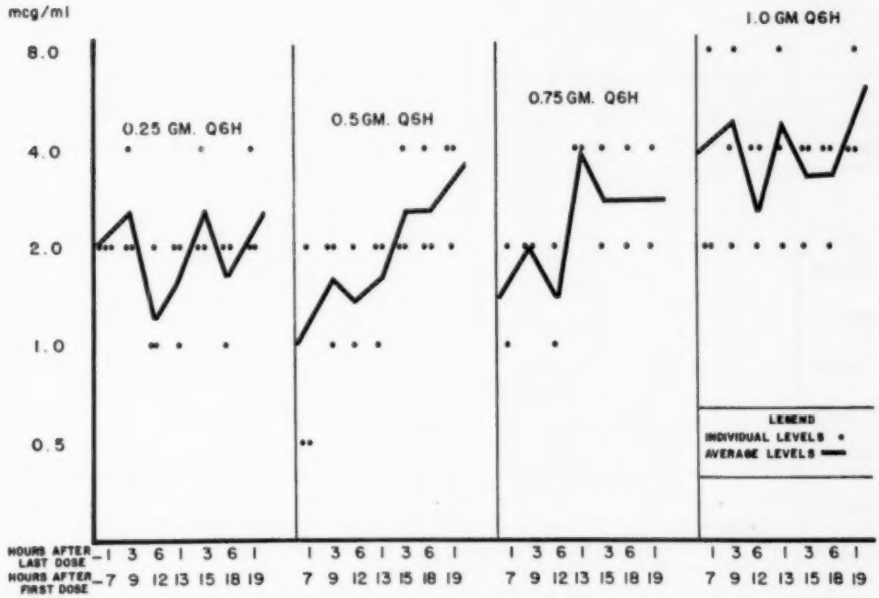

Fig. 4.—Aureomycin HCl in divided doses: individual and average serum levels.

the total amount of aureomycin excreted over the same time interval. The lower recovery of aureomycin may be partly explained by its greater instability in urine.

The cumulative urine excretion of terramycin when given in multiple doses is shown in Fig. 6, and for aureomycin, when given in multiple doses, in Fig. 7.

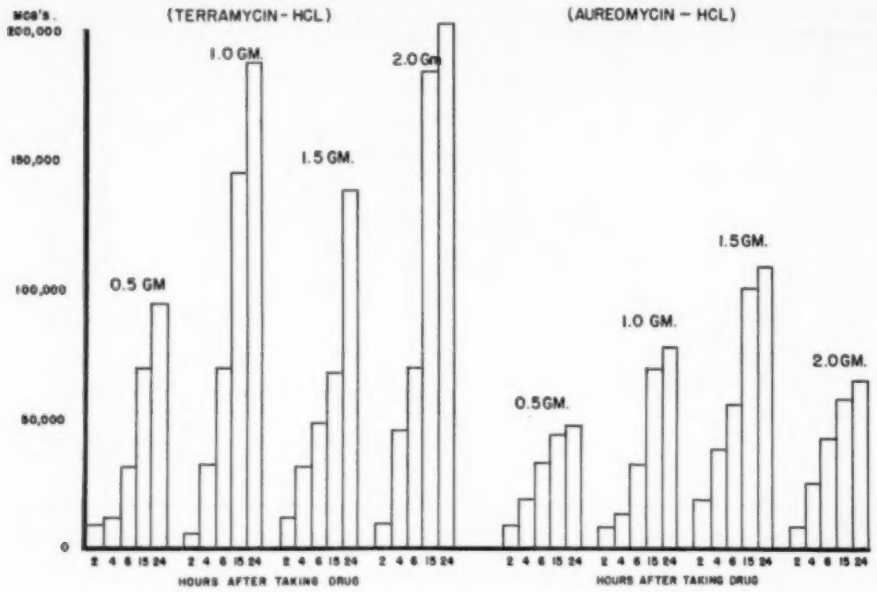

Fig. 5.—Cumulative urinary excretion following a single dose of terramycin or aureomycin.

As in the case of the single dose the total amount of the antibiotic recovered increased with increased dosage and this was greater with terramycin than with aureomycin. Also, as with the single dose, the total amount of drug recovered in the urine is greater in the case of terramycin. Since both terramycin and aureomycin may be used in the treatment of urinary tract infections, it seemed desirable to record the μg./-ml. found in the urine after single doses and divided doses of these drugs. The results are shown in Tables II and III.

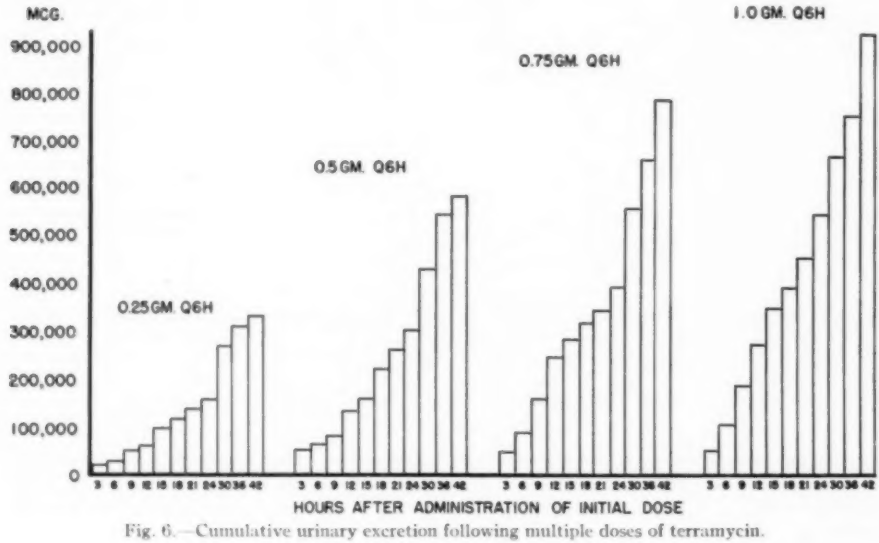

Fig. 6.—Cumulative urinary excretion following multiple doses of terramycin.

TABLE II.—AVERAGE μG. OF ACTIVITY PER ML. OF URINE FOLLOWING A SINGLE ORAL DOSE OF TERRAMYCIN OR AUREOMYCIN

Dose, Gm.	Hours after Dose Was Administered 0–2	2–4	4–6	6–15	15–24
	μG. per Ml. of Urine (Terramycin)				
0.5	26	140	105	97	89
1.0	36	305	328	207	96
1.5	49	116	214	129	44
2.0	39	322	397	298	198
	(Aureomycin)				
0.5	...	97	114	57	0
1.0	...	45	143	85	27
1.5	...	141	218	114	10
2.0	...	62	172	99	26

It can be seen that in the case of terramycin a single dose of 0.5 Gm. produced high urine concentrations for a period of twenty-four hours. In the case of aureomycin at least 2.0 Gm. were required to produce similar concentrations over the twenty-four hour period. In the case of multiple doses the concentration of terramycin was considerably higher than that of aureomycin. It is also noteworthy that the urine concentrations of terramycin twenty-four hours after the last dose were of the same magnitude as those obtained during administration of the drug.

TABLE III.—AVERAGE μG. OF ACTIVITY PER ML. OF URINE FOLLOWING MULTIPLE DOSES OF TERRAMYCIN AND AUREOMYCIN IN MAN

Dose Q 6 Hrs.	Total Dose	Hours after the Initial Dose 3	6	9	12	15	18	21	24	32	40	48
		μG. per Ml. of Urine (Terramycin Hydrochloride)										
0.25	1.0	164	176	105	85	71	113	108	116	185	70	163
0.5	2.0	324	342	40	414	316	359	239	209	475	188	366
0.75	3.0	366	564	255	270	234	243	295	171	262	209	713
1.0	4.0	227	676	754	741	396	400	261	284	617	354	480
		(Aureomycin Hydrochloride)										
0.25	1.0	50	49	36	48	40	37	38	63	29	24	4
0.50	2.0	54	44	36	48	58	123	133	68	160	33	16
0.75	3.0	65	50	25	30	70	70	72	87	63	36	20
1.0	4.0	73	250	148	116	126	97	120	156	165	26	55

DISCUSSION

Preliminary studies of terramycin indicate that its antibiotic activity is very similar to that of aureomycin. As a matter of fact, infrared spectrum analysis shows that although the drugs have definite differences in their molecular structure the fingerprint analysis has many points of similarity. The bacterial spectra of these two drugs also have much in common. It is true that against certain strains of organisms studied terramycin is more active than is aureomycin, but with other strains of the same species the reverse is true. Both drugs in the crystalline state appear to be quite stable, while in solution terramycin is considerably more stable than aureomycin. Both drugs cause some looseness of stools, but this probably can be expected with any oral drug having a wide antibacterial activity and thus causing profound changes in the intestinal flora.

The serum concentrations obtained following comparable single doses of terramycin and aureomycin are for all practical purposes approximately the same. It is of interest, however, that increasing the dose of either drug beyond 1.0 Gm. did not materially increase the blood concentrations. In the case of aureomycin this is quite pronounced and may account for the fact that recent evidence indicates lowering the dose in certain diseases over that previously recommended has not affected the good therapeutic results reported with this drug. Aureomycin given in divided doses again failed to show the expected increase in blood concentration of the drug following increased dosage. As a matter of fact, it appears (Fig. 4) that 0.25 Gm. every six hours is fully as satisfactory as 0.5 Gm. or 0.75 Gm. every six hours, and nearly as satisfactory as 1.0 Gm. every six hours in producing adequate concentrations of the drug in the blood stream. However, with terramycin an increase in dosage from 0.25 Gm. every six hours to 0.75 Gm. every six hours produced a progressive and marked increase in average serum concentrations in the blood. The average blood concentrations of terramycin obtained with 0.75 Gm. every six hours and 1.0 Gm. every six hours are not significantly different.

Following oral doses of 0.5 to 2.0 Gm. of aureomycin and terramycin, considerably more of the latter drug was recovered in the urine over a twenty-four hour period. The lower recovery of aureomycin might be explained by its greater instability but this difference appears too great to be explained on this basis alone.

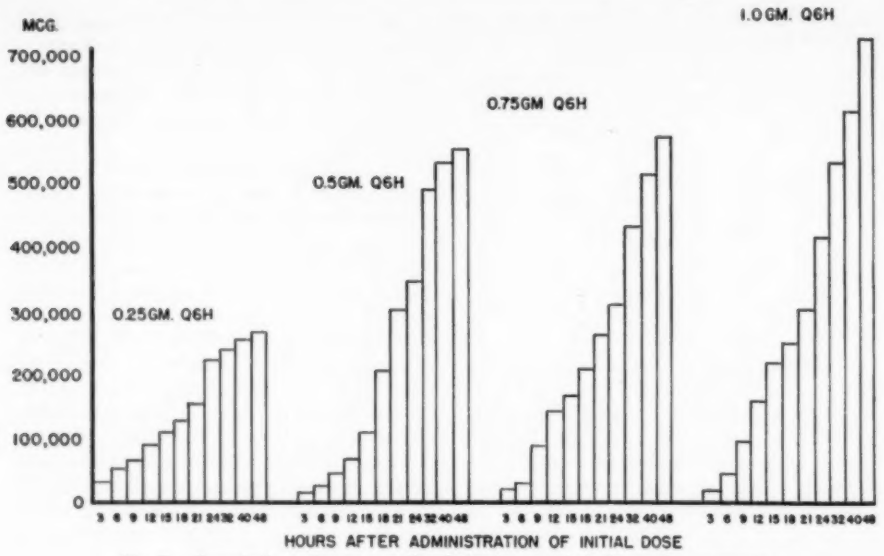

Fig. 7.—Cumulative urinary excretion following multiple doses of aureomycin.

With both aureomycin and terramycin an increase in the oral dose did not in many instances result in a corresponding increase in the blood and urine concentrations. This was particularly true with the higher doses. Since within the lower dose range, increasing the dose results in a corresponding increase in blood and urine concentrations, it would appear that between the low and high doses a point of optimum absorption is reached, and the excess drug is excreted in the feces. Studies are under way to determine the amounts of both drugs excreted in the feces following various oral doses.

SUMMARY AND CONCLUSIONS

1. Terramycin has a wide activity against many bacterial species, both Gram-positive and Gram-negative. It is especially active against the clostridia and (saprophytic) mycobacteria, but has little or no effect on fungi. On the whole the antibacterial spectrum of terramycin is similar to that of aureomycin.

2. Terramycin is readily absorbed from the gastrointestinal tract and produces significant continuous blood concentrations when 250 mg. are given every six hours. The same doses of aureomycin produce comparable blood concentrations.

3. Terramycin is readily excreted by the kidneys and appears in the urine in an active state. The amount excreted depends upon the dose and varies from 10 to 35 per cent of the total amount of drug ingested. By contrast the total amount of aureomycin excreted varied from 5 to 10 per cent depending upon the oral dosage schedule.

4. The concentration of terramycin per milliliter of urine is much higher than that obtained with aureomycin when these drugs are given orally in comparable doses.

5. No significant toxic manifestations were noticed with either drug.

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The Assay of Ipecac*,†

By ANDREW LASSLO and GEORGE L. WEBSTER

The assay of ipecac has been studied and a new procedure involving the process of ion-exchange has been recommended.

THE DEVELOPMENT of the present official method for the assay of ipecac and its preparations may be traced from the earliest gravimetric method of Lefort (1) through the subsequent modifications of Zenoffsky (2), Heikel (3), Lignon (4), Farr and Wright (5), and Keller (6). The last-mentioned worker proposed the procedure which was adopted with little change, except in the choice of indicator, suggested by Herzog (7), in the U. S. P. VIII.

The U. S. P. XIII method differs little from that of the U. S. P. VIII. The differences are in the length of the period of maceration; a change from a mixed solvent of chloroform and ether to ether alone; the use of 0.1 *N* alkali in the place of 0.02 *N* alkali; the use of the milliequivalent of emetine, 0.0240 Gm., in the place of 0.0238 Gm. which was an adjusted equivalent based on the relative proportion of emetine to cephaeline found in Rio ipecac.

The purpose of this investigation was to re-examine the assay of ipecac to determine: (*a*) the completeness and efficiency with which the ether-soluble alkaloids may be extracted from the drug, (*b*) the applicability of an adsorption technique and the optimum conditions for its use for the purification procedure, and (*c*) the accuracy of the end point in the titrimetric determination of the alkaloids. The following description of the method represents the procedure which gave reproducible results on replicate samples.

EXPERIMENTAL

Extraction of the Alkaloids.—Ten grams of powdered ipecac was placed in a 250-ml. dry Erlenmeyer flask and 100 ml. of peroxide-free ether, measured with a 50-ml. pipette, was added. The flask was stoppered tightly with a rubber stopper, the mixture shaken thoroughly and allowed to stand for five minutes. Ten milliliters of a 10% solution of ammonia in water was added and the mixture shaken for one hour in a mechanical shaker. The mixture was allowed to stand for four hours, then shaken again for one hour in the mechanical shaker and, finally, the drug allowed to settle.

Purification of the Alkaloids.—Fifty milliliters of the clear supernatant liquid was transferred by means of a pipette to a 125-ml. Erlenmeyer flask and the pipette rinsed with ether, adding the rinsing to the contents of the flask. The ether solution was concentrated on the steam bath to about 20 ml. and 10 ml. of 0.5 *N* sulfuric acid was added. The ether was completely removed on the steam bath, shaking the flask occasionally.

The adsorption apparatus pictured in Figure 1 was used. The column of adsorbent, 19 mm. in diameter and 135 mm. high, consisted of Florisil 60–100 mesh, uniformly packed with a glass rod, between thin layers of fine glass wool, 0.3 cm. on the bottom and 0.2 cm. on the top of the column. One hundred milliliters of 10% ammonia in water was placed in the separatory funnel. Valve *A* and outlet *B* were opened, outlet *B* being left open until 2 to 3 cm. of liquid covered the solid adsorbent. During the subsequent operations the column was always covered by liquid, using outlet *B* to regulate the height of the covering layer. To speed the flow of liquid, suction was applied through *F* but not to exceed 330 mm. of vacuum; *E* acting as an adjusting valve. After the receiver on the bottom of the column became filled to three-fourths of its volume, valve *E* was completely opened, the suction discontinued, and the receiver emptied by opening valve *C*. (It is important that suction be discontinued and normal pressure be restored in this manner whenever liquid is removed from the receiver.) To restore the rapid flow of liquid through the column, valve *C* was closed, suction applied and adjusted with valve *E*. When about 1 ml. of the ammonia solution was left in the separatory funnel, 200 ml. of distilled water was added in two portions and drawn through the column. When all but the last 20 ml. of water had run from the separatory funnel, a small funnel with a coarse, sintered-glass filtering plate was fitted into the top of the separatory funnel. The solution of the extracted alkaloids in dilute sulfuric acid, obtained as described above, was filtered through this and the flask and the filter washed three times with 30-ml. portions of distilled water. The filter was removed and the separatory funnel and column were washed by drawing through an additional 200 ml. of distilled water. When only 5 ml. of water remained in the funnel, 50 ml. of peroxide-free ether was added and allowed to run through the column until the easily observed line, marking the interface of the ether and water, was about 2 cm. from the bottom of the column. Suction was discontinued, valve *E* opened, and the receiver emptied.

All of the liquid which was collected up to this point was discarded and all of the subsequent eluate collected and combined in preparation for the titrimetric determination of the alkaloids.

One hundred milliliters of an approximately 10% ammonia solution in 95% alcohol was added to the separatory funnel before the last 2 ml. of ether had run out. The alcoholic solution was permitted to run through without suction (valve *E* open) and,

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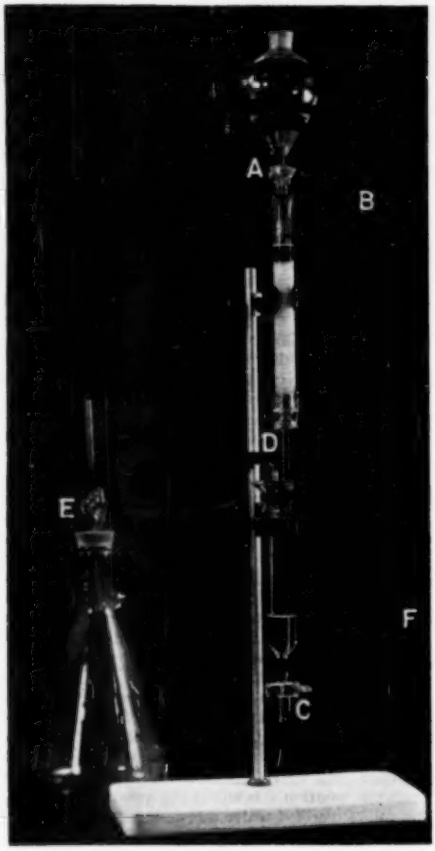

Fig. 1.—Adsorption apparatus used in purification of the alkaloids.

when it had nearly reached the bottom of the adsorption column, the rate of elution was adjusted to 4 ml. per minute by means of valve *A*. After allowing approximately 50 ml. of the alcoholic solution to flow from the separatory funnel, valve *A* was closed and the elution stopped for fifteen minutes. The eluate collected up to this point was removed from the receiver to a 250-ml. beaker and placed on a steam bath to evaporate the solvent. The flow of solvent was resumed at the same rate until all of the ammonia solution had run through the system. The eluate was removed from the receiver and combined with that previously collected The solvent was evaporated almost to dryness.

In these experiments, the same column has been successfully used for three consecutive assays. In that case sufficient ammoniacal alcohol solution was used to provide 100 ml. of the eluate and to keep a small layer of solvent above the adsorbent column at all times. The column was prepared for the next experiment by drawing 100 ml. of 10% ammonia in water through it as described above. It is important that the column never be allowed to become dry if it is to be used in a series of determinations.

Determination of the Alkaloids.—About 30 ml. of peroxide-free ether was added to the residue obtained in the previous stage, rinsing the walls of the beaker during the addition. Exactly 10 ml. of 0.1 *N* sulfuric acid solution was added and the ether was completely evaporated on the steam bath. Fifty milliliters of carbon-dioxide-free distilled water and two drops of methyl red T. S. were added and the excess of sulfuric acid was determined by titration with 0.1 *N* sodium hydroxide solution. The color at the end point was a faint pink which could be changed to a faint orange by 0.05 ml. (1 drop) of 0.1 *N* alkali. The end point color corresponded to that produced in a solution of *p*H 5.2, as determined by a glass electrode *p*H meter.

DISCUSSION

The official procedure directs that the drug and the ammoniacal solvent be shaken either mechanically for one hour or intermittently for two hours before macerating overnight, followed by intermittent agitation for a half hour. It could not be determined whether the direction to macerate for this period of time is specified because it was known that extraction required prolonged maceration or as a convenience for the analyst. It was determined as shown in Table I, samples 16 and 17, that, when one hour of mechanical agitation preceded and followed four hours of maceration, results were obtained in close agreement with those obtained by longer periods of maceration. Attempts to shorten the maceration period to three hours (sample 18) gave significantly lower results.

In February, 1948, Whipple and Woodside (8) recommended maceration of the drug for an overnight period followed by extraction in a Soxhlet apparatus. They used 5-Gm. samples and reported four hours of extraction was sufficient.

Prior to the publication of Whipple and Woodside, experiments of the same kind were completed. It was found that extraction was not complete until it had continued for eight hours. Completeness of extraction was tested at intervals in test experiments by removing 10 ml. of the solution from above the extraction thimble, evaporating the ether, taking up the residue with 0.5 *N* sulfuric acid, and adding Mayer's reagent. Confirmatory experiments (samples 8 to 11) showed excellent precision and the results were considered as representing the best approximation to the actual content of ether-soluble alkaloids in these lots of drug.

Samples of ipecac larger than 5 Gm. were found to require longer periods for complete extraction. Thus, sample 3, which weighed 20 Gm., required extraction for eight hours each day for three successive days before a negative test with Mayer's reagent could be obtained.

The successful use of adsorption techniques in the assay of alkaloid-bearing drugs by others (9–15) directed attention to this type of purification procedure for the alkaloids of ipecac.

A number of exploratory experiments were conducted using several substances that have been used as adsorption materials in chromatography. Activated aluminum oxide, calcium hydroxide, lactose,

TABLE I. ASSAYS OF THE DRUG

Sample and No.	Method of Extraction: Maceration, Hr.	Method of Extraction: Agitation, Type and Hr.	Method of Extraction: Soxhlet, Hr.	Method of Purification	Alkaloids, %
		Rio Ipecac			
1 (*a*)	Overnight	2.0 (*i*) + 0.5 (*i*)	...	U. S. P.	2.41
2 (*a*)	Overnight	2.0 (*i*) + 0.5 (*i*)	...	U. S. P.	2.35
3 (*b*)	48 (*c*)	... ...	24 (*d*)	U. S. P.	2.88 (*e*)
4 (*a*)	Overnight	1.0 (*m*) + 0.5 (*i*)	...	U. S. P.	2.91
5 (*a*)	Overnight	1.0 (*m*) + 0.5 (*i*)	...	U. S. P.	2.89
6 (*a*)	Overnight	1.0 (*m*) + 0.5 (*m*)	...	Florisil	2.99
7 (*a*)	Overnight	1.0 (*m*) + 0.5 (*m*)	...	Florisil	2.97
8 (*f*)	Overnight	1.0 (*m*) + 0.5 (*m*)	8 (*d*)	Florisil	2.97
9 (*f*)	Overnight	... ...	8 (*d*)	Florisil	2.96
		Cartagena Ipecac			
10 (*f*)	Overnight	... ...	8 (*d*)	Florisil	3.16
11 (*f*)	Overnight	... ...	8 (*d*)	Florisil	3.10
12 (*a*)	Overnight	1.0 (*m*) + 0.5 (*i*)	...	U. S. P.	3.06
13 (*a*)	Overnight	1.0 (*m*) + 0.5 (*i*)	...	U. S. P.	3.12
14 (*a*)	Overnight	1.0 (*m*) + 0.5 (*m*)	...	Florisil	3.10
16 (*a*)	Overnight	1.0 (*m*) + 0.5 (*m*)	...	Florisil	3.15
16 (*a*)	4	1.0 (*m*) + 1.0 (*m*)	...	Florisil	3.08
17 (*a*)	4	1.0 (*m*) + 1.0 (*m*)	...	Florisil	3.02
18 (*a*)	3	1.0 (*m*) + 1.0 (*m*)	...	Florisil	2.80 (*g*)

(*a*) 10-Gm. sample, (*b*) 20-Gm. sample; (*c*) intermittently on three successive days; (*d*) until no test for alkaloids; (*e*) average of four determinations on aliquot portions of extract; (*f*) 5-Gm. sample; (*g*) average of two determinations; (*i*) intermittent; (*m*) mechanical.

calcium gluconate, charcoal, and barium sulfate were tried. The ether extract of ipecac was drawn through columns of these materials and attempts made to elute the alkaloids from them with different organic solvents such as alcohol, methyl alcohol, chloroform, ether, acetone, benzene, and several mixtures of these. As adsorbents, activated aluminum oxide and calcium hydroxide had the highest efficiency per cubic centimeter of column. A serious objection to all of this group was that in no case was it possible to elute the alkaloids from the column without simultaneously eluting the resins which had been extracted from the drug and which were also adsorbed by these materials. Since the purpose of the purification procedure was to separate the alkaloids from all other extractives in preparation for the titrimetric determination, it was concluded that none of these adsorbents was satisfactory.

A method which, in principle, should offer a suitable solution to the problem was one making use of ion-exchange. One essential requirement of such a procedure, for this type of assay, was that the eluted alkaloids must be obtained as the amine bases in order that they might be determined titrimetrically. Another requirement of practical importance was that the eluting solvent should be readily evaporated. The first requirement dictated the use of an eluant containing a volatile base and the second requirement, coupled with the solubility characteristics of the alkaloids, required the selection of an organic solvent of high volatility. Furthermore, in the light of the theoretical principles of ion-exchange, the organic solvent should be polar.

Since it was to be expected that the ion-exchange would take place readily from a water solution of alkaloid salts, the original extractive was evaporated in the presence of a small excess of dilute sulfuric acid. This procedure had an advantage in that it precipitated the resins which could be filtered out of the solution before drawing it on the column.

A number of materials were investigated which have been used in ion-exchange columns. The synthetic resin commercially known as Amberlite IR-100 H and the sulfonated coals known as Zeo-Karb H and Zeo-Karb Na proved to be unsuitable because they were appreciably soluble in the alkaline eluant and yielded highly colored solutions. Decalso, a synthetic zeolite, was found to be active as an ion-exchange material and insoluble in the eluting solvent. The adsorptive capacity was shown by comparative experiments to be considerably less than that of Florisil, which was finally adopted as the adsorbent of choice.

As a check on the accuracy of the method of adsorption, elution and titration of the alkaloids in the drug, experiments were conducted using known amounts of emetine hydrochloride and aliquot portions of a solution obtained by dissolving 20 hypodermic tablets of emetine hydrochloride in water to make 200 ml. A comparative experiment was performed using the shaking-out procedure described in the U. S. P. XIII for the assay of Emetine Hydrochloride Injection. The results of these experiments are recorded in Table II. It should be considered that the milliequivalent of the 0.02 *N* acid used in the titrimetric determination of the eluted alkaloid is 4.8 mg. and that there was an uncertainty in detecting the end-point color, when such dilute solutions were used without a comparative color standard, amounting to 0.2 ml. For more accurate work a color standard was prepared using a comparable volume of solution having a *p*H 5.2 to which two drops of indicator solution were added. It was possible to reproduce the color within the limit of 0.1 ml. or 0.5 mg.

The error in the determination of the end-point color using 0.02 *N* sodium hydroxide solution was obvious in the early part of the work. Lowdell (16) discussed the problem of the *p*H of the solution when back titrating with 0.1 *N* sodium hydroxide. He concluded that an accuracy of 0.3% could be obtained by titrating to the color change from red to orange which he determined to correspond to *p*H 4.3. Lowdell quoted results from Baggesgaard-Rasmussen and Wael, who recommended

titration to a full yellow which they reported corresponding to *p*H 6.0 but which Lowdell found to be *p*H 6.3. This disagreement among presumably careful workers made it seem proper that the situation at the end point be examined.

A solution of emetine in 10 ml. of 0.1028 *N* sulfuric acid was titrated with 0.02 *N* sodium hydroxide. The *p*H of the solution was determined with a glass electrode, direct reading *p*H meter after the addition of each ml. of alkali solution and plotted against the volume of sodium hydroxide solution as shown in Fig. 2, broken lines.

For the purpose of comparison a similar curve was plotted for the same amount of the acid containing no emetine. It can be seen from this curve (unbroken line) that the neutrality point, *p*H 7.0, lies midway on the curve between the point where the most rapid change began, corresponding to *p*H 4.8, and where the steep portion began to level off, corresponding to *p*H 9.2.

Similarly examined, the end point of the titration in the solution containing emetine sulfate lies at the midpoint of the steep portion of the curve between *p*H 3.8 and *p*H 6.6, i.e., *p*H 5.2. When two drops of methyl red indicator solution were added to 50 ml. of a solution having a *p*H 5.2, the color of the indicator was a faint but perceptible pink.

The sensitivity of the indicator to additions of 0.02 *N* base and the reproducibility of the color was determined in another way. A titration was conducted without the constant check of the *p*H reading until it was judged that the end point had been reached by a visual determination of the color. The *p*H of this solution was determined to be 4.9. No apparent change in color could be detected until after 0.2 ml. (4 drops) of a base solution had been added. The *p*H of this solution was 5.6 and the indicator was orange in color. Because it was difficult to detect changes in the color with less than this volume of 0.02 *N* alkali it was concluded that equally acceptable results could be obtained by the use of 0.1 *N* sodium hydroxide for the back titration. It was possible to titrate to a point where 0.05 ml. (1 drop) of this solution would change the indicator from its pink color to orange. This change covered the *p*H range in which the true end point lies and could be determined with less indecision than was the case with the more dilute solution of base. In routine assays of the drug, the error introduced by a possible failure to achieve the exact end point could not exceed ±0.03% of ether-soluble alkaloids.

TABLE II.—RECOVERY OF ALKALOIDS

Sample		Alkaloids Calculated, Mg.	Alkaloids Found, Mg.
Emetine 2HCl (crystals)	1.	138.9	139.9 (*a*)
	2.	140.3	140.7 (*a*)
	3.	142.4	143.0 (*a*)
Emetine 2HCl (hypodermic tablets) (*c*)	1.	56.4	56.04 (*a*)
			56.34 (*a*)
	2.	50.0	49.84 (*a*)
			49.59 (*a*)
	3.	50.0	49.92 (*b*)
			49.70 (*b*)

(*a*) Adsorbed and eluted from Florisil column; (*b*) recovered by shaking out with ether from ammoniacal solution; (*c*) twenty tablets dissolved in 200 cc. of solution and calculated aliquots taken for recovery experiments.

SUMMARY

The assay of ipecac has been studied and a new procedure has been recommended.

The extraction of the ether-soluble alkaloids from ipecac has been examined and found to be accomplished with reproducible accuracy in the shortest time by maceration of the drug with ammoniacal ether for a minimum time of four hours preceded and followed by one hour of continuous mechanical shaking.

The classical method of partition between immiscible solvents in the purification procedure was substituted by an ion-exchange process using a Florisil column. The procedure is simple, the apparatus inexpensive and, using multiple columns, one operator may complete the purification and titrimetric determination of the alkaloids in as many as six samples in a four-hour period.

The end point in the titrimetric determination of the alkaloids has been shown to occur at *p*H 5.2. The use of an 0.1 *N* alkali solution was found to yield results which were as acceptable for routine assays of the drug as those obtained with 0.02 *N* solution and with less indecision as to the end-point color change.

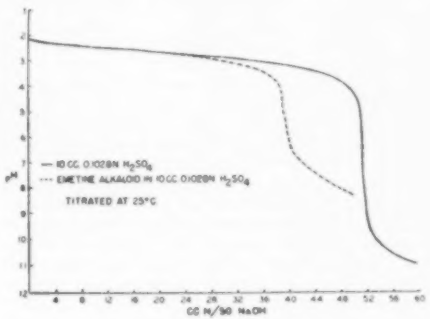

Figure 2.

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Rodent Repellent Studies. III. Advanced Studies in the Evaluation of Chemical Repellents*

By E. BELLACK and JAMES B. DeWITT

In order to bridge the gap between preliminary screening of chemicals for potential rodent repellency and the application of these compounds to paper cartons, more advanced studies in the evaluation of promising materials have been carried out. These studies have resulted in: (1) a modification of the food acceptance technique which eliminates doubtful compounds and also provides a closer analogy to the ultimate goal, and (2) a method for rapidly testing chemicals incorporated in paper. When the results of these latter tests are expressed as a function of time, it can be shown that a distinct correlation exists between the deterrency exhibited by treated paper and the repellency of treated food.

ALTHOUGH IT has been recognized that considerable damage to property and agricultural products results from the activities of other species of rodents, particular attention has been given to the destructive roles of the common brown (Norway) and black rats. In such cases, the amount of damage might be reduced by the use of some substance which would repel the animals, or would deter gnawing activities. The development of such a repellent, or deterrent, capable of application to paperboard cartons and other materials, is the basic objective of the studies on rodent repellents now being conducted by the Fish and Wildlife Service.

The initial phases of these studies, as described in the preceding papers of this series (1, 2), involved the development of a bioassay procedure for measuring repellent activity, and the screening of a large number of materials to determine the repellency associated with certain chemical structures and specific members of various chemical groups. The technique consisted of determining the relative rates of acceptance by laboratory rats of a normal diet and one containing a small proportion (2 per cent) of the candidate material. The results were expressed by an index number (K), which was based upon the differential in acceptance rates on each day of the test period, and on the quantity of the test material, in milligrams per kilogram of body weight, consumed by the test animals.

This technique served as screening medium whereby the majority of chemical compounds were eliminated from further consideration. However, in order that the basic objective might be realized, it was essential that additional tests upon the more active compounds utilize the incorporation of chemicals in paper as the test medium. No suitable procedure for the assay or the evaluation of results was available. Work at the University of Pittsburgh (3, 4) and elsewhere had offered a clue to barrier testing procedures, but the techniques were unsuitable for our purposes, and contained no provision for the interpretation of results. In order to obtain further information on the degrees of repellent activity of certain compounds, and to determine the possible utility of these materials as protectants against rodent damage, a modified bioassay procedure was adopted and additional studies conducted.

EXPERIMENTAL

Food Acceptance Studies.—The technique employed in the food acceptance studies has been described in a previous paper (1). In order to obtain additional information on the repellent activity of test materials, and to further establish the reliability of the test procedure, additional tests were conducted by this technique. In these advanced tests, the candidate repellent was tested at levels in the diet of 2, 1, and 0.5%.

Barrier Evaluation Studies.—[1]In order to further test the efficacy of the repellent materials, they were applied to paper as an impregnant or surface coat, and the treated paper was placed as a barrier between trained rats and a food supply. In order to insure uniformity of results, and to facilitate the experimentation, it was found necessary to modify the available equipment, and to subject the test animals to an intensive training routine.

(*a*) *Construction of Test Cages.*—For the training procedure, a number of special barrier cages were built. These cages, made of hardware cloth, consisted of three sections. In the first section, sufficient space was allowed for a rat to move around freely. The center section contained a frame in which the barrier could be mounted, and the third section, inaccessible except through the barrier, was a duplicate of the first section. In practice, the rat was held in section 1, and food was placed in section 3 to provide the incentive for the animal to penetrate the barrier. Metal doors between the sections were provided to withhold the animal from the center section while barriers were replaced,

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[1] The techniques employed in these studies resemble those employed by Stolurow (3, 4). Some modifications have been introduced in order that the work might be facilitated.

and these also made possible the training of a second animal while the first remained in section 3 eating his "reward."

Although these cages served their purpose admirably, their construction took a considerable amount of time, and space restrictions limited the number which could be maintained. Also, the manipulation of animals and barriers was too difficult for the most expeditious carrying-out of routine experiments. For this latter type of barrier tests a simpler cage was designed. A frame for holding the barriers was cut from galvanized sheet metal so that it would just fit into a standard laboratory rat cage. This frame was provided with flanges which would hold it approximately three inches from the back of the cage, thus furnishing a space for feed behind the barrier without severely restricting the space allotted to the rat in front of the barrier.

(*b*) *Training of Experimental Animals.*—Animals were chosen at random as to sex and coat color, but early experiments indicated that a minimum weight of 200 Gm. was necessary for the purpose. First, rats were placed in section 1 of the training cages, and food was placed in section 3. After a few feedings the animals became accustomed to receiving food in section 3 and were then ready to start through the following training steps:

1. Barrier consisting of single thickness of Kraft paper pierced with a $^1/_4$-inch hole located 1 inch from the bottom of the barrier. Each animal pierced two such barriers before passing to the next step.
2. Barrier consisting of 5 thicknesses of Kraft paper pierced with a $^1/_4$-inch hole located 1 inch from bottom of paper.
3. Barrier consisting of a single thickness of Kraft paper pierced with a $^1/_4$-inch hole.
4. Barrier consisting of single thickness of Kraft paper (no opening). Each animal pierced two such barriers before passing to the next step.
5. Barrier consisting of three thicknesses of Kraft paper (no opening).
6. Barrier consisting of 5 thicknesses of Kraft paper with $^1/_4$-inch hole.
7. Barrier consisting of 5 thicknesses of Kraft paper (no opening).
8. Barrier consisting of 8 thicknesses of Kraft paper (no opening).
9. Barrier consisting of 5 thicknesses of Kraft paper with $^1/_4$-inch hole.
10. Repeat 5 and 7.
11. Barrier consisting of single thickness of Kraft paper coated on both sides with synthetic latex.
12. Repeat 4 and 7.
13. Barrier consisting of 5 thicknesses of Kraft paper laminated with synthetic latex.
14. Barrier consisting of 5 thicknesses of Kraft paper laminated and coated with synthetic latex.
15. Repeat any of above when required.

Before beginning this training procedure, the animals were starved for twenty-four hours, in order to furnish the necessary hunger drive, then placed in section 1 of the training cage fitted with the barrier, and allowed to remain until they had pierced the barrier and had consumed the food placed in section 3 as their "reward." In each case, animals were rested for twenty-four hours following the successful completion of a step. The amount of food given as the "reward" for successful penetration of a barrier was purposely restricted so that sufficient hunger-drive would be present when the animal was ready for the next step. Animals were given unlimited food over the weekends, then starved for twenty-four hours prior to resumption of training. Animals which did not progress as rapidly as the average were given extra training, and where this did not avail, they were discarded. At the end of about six weeks of training, the rats were so conditioned that all would penetrate barrier No. 14 within ten minutes of the time they were placed in the training cage. This accomplishment is noteworthy, considering the fact that untrained rats actually starved when separated from food by a single thickness of paper. These trained animals continued to pierce barriers readily for a year or more, after which time they were replaced with freshly trained animals.

(*c*) *Preparation of Barriers.*—Various methods for the construction of paper barriers have been used. The simplest barrier consists of a single thickness of Kraft paper cut in the shape of a square 6 inches on a side. Notches are cut in it to provide for the bolts of the holder. More complicated barriers are built up of layers of Kraft paper laminated with synthetic latex, and the composite notched to fit the holder. The test compound was added to the barrier either by impregnation of the paper of one or more of the layers, by dusting on a lacquer surface, or by coating the barrier surface with a solution or suspension of the material in lacquer. Various other methods of treating the barriers were tried from time to time.

RESULTS AND DISCUSSION

Since any procedure for barrier testing must of necessity be time-consuming, it is desirable that the screening out of less promising materials be carried to the maximum degree. The food acceptance technique has proved to be the most effective means for this preliminary work, with one possible drawback. In the expression of repellency by the "K value," an abstract quantity, our sole measure of accuracy is the degree of reproducibility of results. If it can be shown that the results of any one test which gave a comparatively high K value can be substantiated by subsequent tests, it may be assumed that the results are reliable, and that our screening method is satisfactory, i.e., that it offers a measure of repellent activity. As a further test on the reliability of "K values," feeding tests were carried out in which the concentration of test material in the diet was lowered successively to 1% and 0.5% (tests at 0.25% gave misleading results) and the K values at the three levels compared for possible variations exceeding those indicated by a table of standard deviations. These "repeat" tests serve two purposes—the substantiation of earlier results and the provision of a closer analogy to the ultimate goal. Since the amount of chemical which can be incorporated in paper is very small, the feeding tests at the lower levels will indicate which chemicals may be expected to retain their repellency when present in the concentrations most nearly approximating those used in the manufacture of treated paper.

Table I lists the results from repeat tests on a representative number of compounds. Values which show a range exceeding the normal variation are indicated in the table by an asterisk. (Right hand column.)

Table I shows that compounds giving the highest initial K values, i.e., between 91 and 100, give the most consistent results on repeat tests, while compounds giving lower initial K values showed progressively poorer consistency. Similarly, the minimum variations occurred in compounds in the highest range and the maximum variations occurred in compounds in the lowest range. Table II lists examples from Table I which gave minimum and maximum variations in K values.

TABLE I.—EFFECT OF VARIATION IN TEST LEVELS UPON REPELLENCY INDICES

Compound Code No., DR-	K Values Obtained at Dietary Levels of 2%	1%	0.5%
A. Compounds Having Original K Values Below 80			
1868*a*	67.7 (2)[a]	78.6	72.8
1873	67.4	74.5	75.7
1884	−27.0 (2)	0	55.0*
1886	− 2.9 (2)	44.0	65.4*
1889	72.6 (2)	72.1	88.6*
1899	73.7 (2)	85.6	83.1
1902	77.9 (2)	79.3	87.7
1928	79.1 (2)	83.5	90.0*
1988	80.0	73.0	98.8*
Proportion of compounds showing normal variation in K value—44.4%			
B. Compounds Having Original K Values between 81 and 90			
1874	83.2 (2)	79.2	92.7*
1883	84.7 (2)	87.4	88.0
1900	81.8 (2)	83.3	88.2
1903	85.1 (2)	80.3	76.0
1939	81.3 (2)	71.6	74.2
1942	81.7 (2)	40.5	73.3*
1968	89.5	88.3	80.6
1991	89.8	89.3	89.9
1990	90.0	99.2	66.0*
Proportion of compounds showing normal variation in K value—66.7%			
C. Compounds Having Original K Values between 91 and 100			
1863	100	99.0	99.5
1866	98.0 (2)	99.0	99.5
1867	94.6 (2)	92.0	86.9
1868	98.8 (2)	97.8	
1875	100 (2)	98.0	99.5
1881	100 (2)	100	96.1
1896	93.3 (2)	92.3	93.7
1919	97.6 (2)	90.9	90.4
1904	95.8 (2)	93.0	83.0*
1921	100 (2)	98.7	98.1
1924	100	95.6	88.2
1946	97.5 (2)	94.7	
1953	91.1 (2)	94.2	93.2
1955	95.9 (2)	95.4	89.4
1957	90.4 (2)	80.5	79.2*
1958	94.5 (2)	94.0	93.3
1960	100 (2)	98.3	99.2
1961	93.2 (2)	95.1	95.1
1965	100	86.0	91.0
1966	100	94.6	95.3
1972	100 (2)	100	99.8
1973	95.5 (2)	99.2	99.1
1976	96.4	100	99.8
1977	98.8	98.8	95.8
1978	95.3	89.3	82.5*
1983	90.1	86.0	89.9
1986	90.5	100	100
1993	94.5	89.5	92.3
1994	100	98.5	99.0
1996	100	96.9	98.5
1997	94.2 (2)	100	98.1
2000	92.0 (2)	98.7	94.4
Proportion of compounds showing normal variation in K value—90.6%			

[a] Figures in parentheses indicate number of tests at the 2% level, and values at this level are averages.

TABLE II.—RANGE OF VARIATION IN K VALUES FROM REPEAT TESTS

Compound Code No., DR-	K Values Obtained at Dietary Levels of 2%	1%	0.5%	Range
A. Maximum Variation				
1884	−27.0	0	55.0	82
1886	− 2.9	44.0	65.4	68.3
B. Minimum Variation				
1972	100	100	99.8	0.2
1863	100	99.0	99.5	1.0

Early experiments proved that a barrier consisting of a single sheet of paper, regardless of the amount of candidate repellent present, offered no deterrent to the rat. The animal was able to cut through the paper with his incisors without coming into contact with an appreciable amount of the chemical. For this reason barriers were constructed of five or more layers of paper laminated with a strong adhesive (synthetic latex) so the rat would be forced to spend considerable time in contact with the barrier in order to penetrate it, and thus the chemical would be able to exert its full effect. The method of inclusion of the chemical also had a strong bearing on the rate of penetration. When the material was coated on or impregnated in one of the inside sheets, the animal was much more likely to penetrate the barrier readily than if the chemical were all coated on the outer (exposed) surface. The figures in Table III illustrate the difference in penetration time of different types of barriers.

As a result of these and similar experiments, most succeeding barriers were constructed so that the

TABLE III.—PENETRATION TIMES FOR DIFFERENT TYPES OF BARRIER CONSTRUCTION

	Time (min.)
Compound DR-1560 (Repellency Index K = 92); (Control time 41.2 min.)	
Compound dusted on lacquered surface (0.05 Gm./sq. in.)	139.6
Compound mixed with lacquer and painted on (0.05 Gm./sq. in.)	87.0
Compound DR-1432 (Repellency Index K = 88); (Control time 11.3 min.)	
Compound dusted on lacquered outer sheet (0.05 Gm./sq. in.)	86.8
Compound dusted on lacquered second sheet (0.05 Gm./sq. in.)	47.6
Compound dusted on lacquered 2nd and 4th sheets (0.10 Gm./sq. in.)	61.7

candidate material was concentrated on the surface exposed to the rat. The control time noted in Table III refers to the average time for penetration of a set of barriers similar in all respects to the treated barriers except that no chemical is included. For every group of treated barriers tested at any one time, a set of these control barriers is tested so that a comparison may be set up. As a general rule, five barriers of each type are included in a group, and the time for penetration is the average of these.

The experimental procedure having been decided upon, the next step was to find a factor which would express concisely and accurately the deterrent value of a particular barrier. Among the suggested variables which such a factor might contain are: time required to pierce the treated barrier, time required to pierce a similar untreated barrier, weight of animals piercing treated barriers, weight of animals piercing untreated barriers, concentration of chemical in treated barrier, area of contact (area of penetration), and sex and coat color of the animals. Numerous experiments have shown that these last two have little if any effect on the rate of penetration. The area of contact seems constant for almost any rat weighing between 200 and 400 Gm., and Fig. 1 illustrates the effect of weight on penetration time.

As can be seen from this chart, the effect of animal weight is within the range of standard deviation and thus is of no consequence. Our deterrent factor, D, then becomes:

$$D = \frac{t_1}{t_2\, c} \qquad (1)$$

where t_1 is the average time required to pierce the treated barriers, t_2 the time required to pierce similar untreated barriers, and c is the concentration of chemical in the treated barriers. This equation is based on the assumption that the speed of penetration is inversely proportional to the concentration of chemical present. Experiments have shown that this is roughly true. However, D is now expressed in units of inverse concentration, since the time units cancel. It thus becomes apparent that our equation is lacking in figures containing weight units and area units. The obvious remedy for this situation is to include the average weight of animals piercing treated barriers and the area of penetration. The inclusion of the weight figures may be justified by considering the fact that the repellency of a chemical should be expressed as a ratio of weight of chemical to weight of animal, just as it is in the evaluation of feeding tests. The inclusion of area of penetration needs no justification, since it may be considered a constant and will have no varying effect. The final equation for D is:

$$D = \frac{t_1 W_1}{t_2\, A c} \qquad (2)$$

where the t's and c are as in Eq. (1), W_1 is the average weight of animals piercing treated barriers, and A is the area of penetration. Time may be most conveniently expressed in minutes, weight in kilograms, area in square inches (2 sq. in. may be accepted as the constant value for A), and concentration in Gm/sq. in. All units will cancel to give an abstract number for D. The time element itself has posed a serious problem in the light of recent

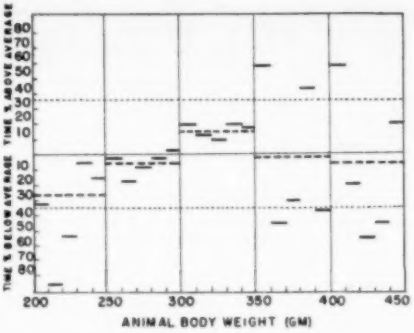

Fig. 1.—Effect of animal weight on penetration time for all barriers.

developments. It was found that if a rat did not attack the barrier immediately, the likelihood of any later penetration became extremely doubtful. Therefore it was deemed advisable to replace the animal if he failed to pierce the barrier within sixty minutes of the time he was placed in the cage. On the more deterrent barriers, as many as ten animals have been required before penetration is completed, and the time will have to include the total time of exposure of the barrier to all the animals involved. Thus t_1 will be the average of these total times, and W_1 will be the average of the average weights.

In the evaluation of the data from barrier tests, it must be remembered that it is impossible to obtain standardized barriers under the conditions of preparation, and that the number of animals employed in any test may represent an inadequate sample of the population. However, inasmuch as the compounds used in these tests have been evaluated by the food acceptance technique, for purposes of comparison they may be divided into groups corresponding to the repellency indices. On the basis of the expected variations in the values for K, as indicated by the standard deviations, it appears logical to group together those compounds having K values between 91 and 100; those having K values between 81 and 90; and those having K values below 80. The results may then be shown in two ways: as differential factors showing the relative times required for the penetration of treated versus control barriers, and in terms of the deterrency factor (D) defined above. The results obtained by these methods are shown in Table IV.

On the basis of the data presented in this table, it appears that a definite correlation between food acceptance repellency and barrier deterrency exists. Despite the fact that the data are not consistent for all compounds within each group, it can be seen that the average penetration time differential increases with increases in the values of K, and thus the D value increases.

TABLE IV.—CORRELATION BETWEEN BARRIER DETERRENCY (*D*) AND FOOD REPELLENCY INDICES (*K*)

Code No., DR-	*K* Value	No. Barriers Tested	*F*[a]	*D*
	A. Compounds Having *K* Values Below 80			
2014	43	5	0.83	2.65
1557	52	5	1.50	4.06
1893	56	5	2.52	8.10
2022	67	5	1.14	3.60
2024	71	5	0.36	0.92
2015	76	5	0.89	2.90
	Total	30	Av. 1.21	Av. 3.71
	B. Compounds Having *K* Values between 81 and 90			
1630	82	5	1.50	4.65
1631	82	5	1.71	4.22
1587	82	3	10.35	29.40
2056	82	5	1.85	5.86
1628	83	2	2.51	6.57
2048	83	4	2.40	7.10
1639	85	4	3.28	10.25
1608	86	4	4.30	11.79
1773	86	5	2.42	7.12
1566	86	3	3.93	11.35
2038	86	4	3.32	10.10
2001	87	2	9.90	32.70
1610	88	3	3.66	11.88
1609	88	3	4.73	13.80
1432	88	5	7.69	21.30
1553	89	5	3.12	9.83
1590	89	3	1.73	4.79
1563	90	7	2.14	4.13
1580	90	5	12.72	37.25
1636	90	5	7.02	21.65
1591	90	5	4.90	14.20
1983	90	5	2.42	8.20
	Total	92	Av. 4.44	Av. 13.10
	C. Compounds Having *K* Values between 91 and 100			
2051	91	5	4.77	16.20
1552	91	5	1.98	6.90
1560	92	5	3.37	10.30
1605	93	3	5.70	20.80
1593	95	5	3.90	10.23
1551	96	5	1.60	5.26
1976	96	1	12.00	35.80
1919	97	5	5.20	14.90
2149	98	3	4.24	12.20
1972	100	2	3.48	10.10
1960	100	2	7.30	21.80
1966	100	5	2.90	9.45
1866	100	10	18.05	72.80
1875	100	5	7.20	26.05
1774	100	5	7.50	22.85
	Total	66	Av. 5.95	Av. 19.68

[a] $F = \frac{\text{Av. penetration time for treated barriers}}{\text{Av. penetration time for control barriers}}$

The inconsistency in some of the data from barrier tests is a troublesome factor. For example, one compound with a *K* value of 96 gave a *D* value only slightly higher than that obtained from a compound with a *K* value of 52. Possible reasons for such discrepancies are the lack of uniformity in the preparation of the barriers, and individual variations in activity found in small groups of test animals. Up to the present time, all barriers have been made by hand, and it is probable that the construction of the barriers varies enough to cause some of the variations in penetration time. It is also quite likely that the method of inclusion of the chemical in the barrier will have to be specific for each material used, since it has not been established by any means that all materials are repellent for the same reason, and thus may be utilized in the same manner. This and other similar problems must be solved in the next step in a logical procedure to find a practical repellent. Such a step will involve the participation of the paper industry in preparing special treated papers for evaluation in barrier and field testing.

SUMMARY

Food acceptance studies for the determination of rat repellency have been modified to include tests in which the concentration of candidate repellent is varied from 2 per cent to 0.5 per cent. Of the total of 290 chemicals which have been subjected to these repeat tests, 57 may be eliminated from further consideration because of lack of consistent behavior. By groups based on initial K values, compounds scoring below 80 were consistent in 50 per cent of the tests; from 81 to 90, in 77.4 per cent, and from 91 to 100, in 87.4 per cent.

A procedure and a method for the numerical expression of results have been devised for the evaluation of the deterrent effects to white rats of chemicals when incorporated in paper barriers. A distinct correlation has been shown to exist between barrier deterrency and food repellency.

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A Phytochemical Study of *Xanthium canadense**,†

By NORBERT R. KUZEL‡ and C. E. MILLER§

It has been found that hydroquinone is responsible for the toxic effects produced by the ingestion of various species of Xanthium. A method of isolation of the toxic principle is discussed as well as the preliminary report on the toxicology of hydroquinone. A comparative chemical study of the kernel and the bur is also included.

SINCE 1880 a controversy has existed concerning the toxicity of cocklebur. A survey of the literature showed that there have been innumerable cases of reported poisonings of cattle, hogs, and sheep by cocklebur. Zander (1) even reports several cases of human poisoning attributed to cocklebur. There are almost as many reports in the literature that Xanthium is completely innocuous.

Zander was the first man to make a comprehensive study of the toxicity of cocklebur and he concluded that his work revealed no trace of a toxic principle. Rostelli and Gibelli (2) state that the plant contains an alkaloid which exerts a strong action on the central nervous system. They were unable, however, to isolate this alkaloid. Krantz and others (3) report that an extract of Xanthium burs was found to be innocuous when administered to *Maccus Rhesus* monkeys. He attributed the depressor response to choline which was identified. Chopra and associates (4) found a soluble glycoside to be present which was inert physiologically. Compore and his coworkers (5) found the toxic principle to be a saponin. Recently Carr (6) has reported that the oil obtained from *Xanthium spinosum* is pharmacodynamically inert.

There are many other cases of reported poisonings by Xanthium that are too numerous to mention. In North Dakota many cases of stock poisoning have been reported to local veterinarians. In view of the prevailing confusion regarding the apparent toxicity of this plant, further investigation seemed advisable.

EXPERIMENTAL

Chemical Study.—Several reports of chemical analyses have been published but considerable variation of results has been noted. In addition, no comparative chemical study of the kernel and bur has been published. Therefore it was decided to make a comprehensive study of the chemical constituents of the bur and kernel. The procedures used for this study were either official in the United States Pharmacopoeia XIII or were outlined in the Association Official Agricultural Chemists Fifth edition. The results are tabulated in Table I.

Preliminary Investigation.—The plant material used for this work was obtained from several sources in North Dakota. The burs were identified as belonging mainly to the species *Xanthium canadense*.

A hydroalcoholic extract was prepared and processed for alkaloids by the official method of the United States Pharmacopoeia. Negative results were obtained with the several alkaloidal reagents.

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† This paper is based on a thesis submitted by Norbert R. Kuzel, American Foundation for Pharmaceutical Education Fellow, to the Graduate Council of North Dakota State College in partial fulfillment of the requirements for the degree of Master of Science.

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TABLE I.—COMPARATIVE CHEMICAL ASSAY OF XANTHIUM

Assay	Bur, %	Kernel, %
Ash (total)	5.45	6.05
Acid insoluble ash	0.79	1.48
Total nitrogen	2.20	6.83
Crude protein	13.75	42.76
Moisture	6.71	8.34
Crude fat	17.08	32.80
Crude fiber	53.59	2.75
Carbohydrate (total)	1.56	4.71
Reducing sugar	0.00	0.00
Invert sugar	1.25	3.36
Starch	0.31	1.35
Alcohol-soluble extractive	28.32	47.06
Diluted alc.-soluble extractive	8.64	9.32
Petroleum benzin extractive	15.71	29.23
Volatile ether-soluble ext.	8.37	12.70
Non-volatile ether-soluble ext.	8.63	20.04
Water-soluble extractive	5.76	8.44
Volatile and undetermined prin.	1.95	2.59

A 10% aqueous extract of the kernels was prepared and tested for toxicity on mice. The extract was found to be toxic. A Molisch reaction did not show the typical purple ring but a characteristic bright green coloration formed in the acid layer. Reduction tests were obtained with Folin-McElroy reagent, Benedict's and Fehling's solution. As a result of these reduction tests an attempt was made to isolate the agent responsible for the reactions observed.

Bay-Gisvold Extraction Method.—From a review of the literature, it was decided to use the Bay and Gisvold method (7) of extraction. About 15 Gm. of the kernel was placed in a Waring Blendor and 150 cc. of water was added, blended for ten minutes, heated to 70°, and filtered. The filtrate was partially evaporated under vacuum, saturated with sodium sulfate and 2 Gm. of charcoal added. The material was then dried *in vacuo* over sulfuric acid, placed in a Soxhlet extractor and extracted with furan for about twenty hours. A yellowish crystalline material was obtained. The substance was recrystallized from ether, washed with chloroform to remove the color, and tested for toxicity. The material proved to be quite toxic to experimental animals as well as displaying the same toxic pattern as found in the field. Further extraction with various solvents was attempted but it was found that furan[1] and tetrahydrofuran[1] produced relatively pure crystalline deposits.

Characterization of the Crystalline Material.—The melting point of the substance was found to be between 168° and 169°. Mixed melting points composed of several lots showed no depression. The crystals were very soluble in ether, ethanol, and benzin. The material was soluble in cold water about five parts per hundred, very soluble in hot water, and insoluble in chloroform. With sulfuric acid a rust-brown color was formed. With nitric acid a bright green color was produced, accompanied by a vigorous evolution of the oxides of nitrogen. The Molisch reaction still showed only the green coloration in the acid layer. An aqueous solution when exposed to air and light turned a dark amber—this reaction being hastened by the addition of alkali.

Identification of the Crystalline Material.—By means of sodium fusion it was found that no nitrogen, sulfur, phosphorus, or halogens were present. Reduction tests on copper solutions were all positive but attempts to form osazones, 2,4-dinitrophenylhydrazones or semicarbazones were unsuccessful. The material was optically inactive. The *p*H of a 1% aqueous solution was found to be 6.88 as compared to a reading of 6.9 for the solvent. There was no reaction with Schiff's reagent. The Folin-Denis test (8) for free phenolic groups was positive. From the above data, a quinone-type structure was suspected. It was observed that hydroquinone possessed the same physical properties as the unknown and by experiment it was found that it possessed the same chemical properties. A spectrophotometric examination of an alcoholic solution produced a very characteristic curve with maxima at 225 and 292mμ, plotting optical density against wave length. A solution of purified hydroquinone in alcohol showed the same curve and maxima points.

Anal.: Calcd. for $C_6H_6O_2$: C, 65.6; H, 5.47. Found: C, 65.3; H, 5.44.

Pharmacology.—Marsh (9) and others have described the toxic symptoms as found in the field cases of poisoning by Xanthium. In the laboratory these toxic symptoms were reproduced and elaborated by the injection of aqueous extracts, isolated crystalline material, and pure hydroquinone. The symptoms were observed on several experimental animals with varying symptomology. Mice, when injected intraperitoneally, showed a marked irritation at the site of injection, accompanied by a marked diuresis. The body became filled with tremors, slowly turning into coordinated hopping movements culminating in a frenzied running about the cage. The animal became ataxic and rolled over on its side but still continued its running movements. Just before death the animal became spasmodic, relaxing after death. The symptoms in the pig showed initial depression, accompanied by a bloody foaming at the mouth, repeated urination, tremors of the body, dyspnea, and the characteristic running movements followed by death. It was found that oral administration generally increased the fatality. A tabulation of approximate lethal doses is presented in Table II. The figures listed are only approximate because of the limited number of test animals available.

Autopsy Findings.—The autopsies revealed smal individual differences but in general the pattern was the same. There were innumerable subcutaneous and muscular hemorrhages, the lungs were generally collapsed and off color, the liver was congested with localized hemorrhages, the spleen and kidneys were

TABLE II.—COMPARATIVE TABLE OF LETHAL DOSAGES

Animal	Lethal Dose in Mg./Kg. of Body Weight
Mouse	250
Guinea pig	150
Pig	175
Chick	375
Turkey	>400

[1] Acknowledgment is given to E. I. du Pont de Nemours & Co., Inc., for generously supplying the furan and tetrahydrofuran used.

engorged with blood and fluids and numerous petechial hemorrhages were observed, the intestines were red and congested, and various abnormalities of the heart were seen. There was heavy serious infiltration of all body cavities.

DISCUSSION

It has been demonstrated that free hydroquinone is the toxic principle which has been responsible for the numerous cases of poisoning, but some question is still present as to whether the hydroquinone is found free or in combination in the plant. The possibility exists that it may occur in glycosidic combination similar to the well-known arbutin. For this reason various methods of extraction were tried and the material was treated in different ways before extraction, but only free hydroquinone was obtained. If the material is autoclaved before extraction, instead of the usual amber filtrate, a colorless filtrate is obtained which is characteristic of the hydroquinone form rather than the oxidized amber quinone form. Thus it would seem likely that a heat labile oxidase enzyme is present in the plant to oxidize the hydroquinone to the much more toxic quinone. No attempt was made to develop an antidote but as a preliminary step a report of Seremin (10) proves interesting. He reports saving the lives of rabbits in convulsions from hydroquinone by the injection of calcium chloride.

CONCLUSIONS

1. It was found that the toxic properties of the various species of Xanthium was due to hydroquinone.

2. The hydroquinone was found free, but there is still a possibility that at certain stages of plant growth it may exist as a glycoside similar to or identical with arbutin.

3. The historic toxicological picture of Xanthium poisoning found in the field has been reproduced in the laboratory by the administration of hydroquinone.

4. A tabular list of the chemical components of Xanthium is included in this paper.

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Chemical Investigation of the Volatile Oil of *Litsea guatemalensis* Mez.*

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The physical and chemical characteristics of the volatile oil obtained from *Litsea guatemalensis* Mez. have been determined. Purification and characterization of various fractions obtained upon fractional distillation of the oil under reduced pressure yielded 1,8-cineole, *dl*-carvone, evidence for linaloöl, and indication of a hydrocarbon. The oil was found to contain 49.6 per cent of 1,8-cineole.

Litsea guatemalensis Mez. is a shrub or small tree, seldom more than six meters high, which is indigenous to Jalapa, Sacatepequez, Chimaltenango, and Guatemala, and is found in dense, moist forests, as well as in open pine forests and in thickets, at altitudes of 1500 to 3150 meters. A complete botanical description of the plant has been given by Standley and Steyermark (1). The leaves of *L. glaucescens* HBK. and other species are said to have an aromatic odor similar to that of bay leaves. They are commonly used by the natives of Guatemala for flavoring foods, especially soups and meats.

An extensive literature survey indicated that there had been no previous investigation of the volatile oil obtained from *Litsea guatemalensis* Mez. The investigation reported here was carried out on a sample of the oil obtained from Keilhauer, Pagran and Co., Guatemala. The examination involved a determination of the physical and chemical characteristics of the oil, separation of its components by fractional distillation, determination of purity of the fractions by the application of thermal analysis, characterization of the purified fractions by physical and chemical means, and determination of the percentage composition of some of the identified components in the oil. It was anticipated that thermal analysis could be used to ascertain the

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purity of the various fractions resulting from distillation of the oil. However, it was necessary to impose certain limitations throughout the investigation since the amount of oil available was limited to the original shipment of 900 cc., its production having been discontinued because of the lack of a market in this country.

The presence of 1,8-cineole and of *dl*-carvone in the oil was definitely established and a number of derivatives were prepared from each of these compounds. The presence of linaloöl was demonstrated, and the presence of a hydrocarbon was indicated, but no crystalline derivatives of it could be prepared.

EXPERIMENTAL

Physical Characteristics.—The oil as received was yellow in color and possessed a sharp camphoraceous odor. The following physical constants were determined: $n_D^{26.1^\circ} = 1.4690$ ($n_{corr.}^{25} = 1.4694$); $[\alpha]_D^{25} = -10.01^\circ$; $d_{25}^{25} = 0.9174$. Table I shows the boiling point range of the oil at atmospheric pressure (745.8 mm.). Fractions 1 to 3 in this determination contained water, and the low temperature recorded was due to the fact that at this point the distillation was effectively a steam distillation. All fractions decolorized a solution of bromine in carbon tetrachloride as well as aqueous potassium permanganate.

TABLE I.—BOILING POINT RANGE OF OIL OF *Litsea gualemalensis* MEZ.

Fraction	B. P., ° C.	$n^{25.2}$
1	97–100	1.4570
2	100–112	1.4550
3	112–114	1.4541
4	114–154	1.4573
5	154–160	1.4602
6	160–168	1.4618
7	168–172	1.4635
8	172–176	1.4680
9	176–190	1.4810
10	190–205	1.4856
11	205–220	1.4880

Thermal analysis of the oil showed no thermal breaks, a constant series of solid solutions evidently being obtained.

Chemical Characteristics.—The oil burned with a smoky flame, leaving no residue. Qualitative analysis showed the absence of nitrogen, sulfur, and halogens. The oil immediately decolorized a solution of bromine in carbon tetrachloride with no detectable liberation of HBr and immediately decolorized 2% aqueous $KMnO_4$.

With phenylhydrazine a slight cloudiness developed after standing one-half hour, and with 2,4-dinitrophenylhydrazine clouding developed upon standing for twenty minutes. Ferric chloride gave a negative phenol or enol test. With Lucas reagent the oil blackened with the production of a great deal of heat. With metallic sodium there appeared to be a slight evolution of gas, with the mixture warming slightly. With acetyl chloride the oil blackened within five minutes and a slight amount of heat developed.

In an attempt at quantitative estimation of the aldehyde and ketone content of the oil, using sodium sulfite solution and a cassia flask in the usual manner, approximately 0.5 cc. of the 10-cc. sample of oil went into solution in the aqueous layer. In addition, however, there appeared a dark yellow layer, amounting to 0.30–0.35 cc., between the bisulfite solution and the unreacted oil.

In an attempt to determine the saponification value of the oil, darkening of the saponification mixture was so great that titration of the excess alkali could not be accomplished.

Fractional Distillation.—Distillation of the oil under reduced pressure was carried out in a fractionating system, the component parts of which were obtained from the Ace Glass Company. The column was 36 inches long, ½ inch i.d., vacuum jacketed, silvered, and indented, with spherical joints at top and bottom and observation stripes at the front and rear, extending the full length of the column. The distilling head was equipped with a pivoted funnel, operated by an electromagnetic coil, and provided with a ground glass joint at the side for the thermometer. The fractometer used had independent cycles for take-off and reflux time, with a maximum time of one hundred and twenty seconds for each cycle. The receiver was equipped with stopcock control and an outlet for vacuum operation, and was connected to a special vacuum receiver which was designed to collect 11 fractions without breaking the vacuum in the entire system.

The fractionating column was suitably insulated against loss of heat by surrounding it with two glass cylinders, the inner one of which was wrapped with two coils of Driver-Harris nichrome resistance wire, 22 B and S gauge. In order to prevent condensation of the vapors before reaching the condenser, which would result in total reflux, the distilling head was also provided with a heating coil. Three junction copper-constantan thermocouples were provided for the pot and for the bottom, middle, and top of the column, as well as for the distilling head. Readings were taken with a Rubicon portable potentiometer, the cold junction being immersed in an ice bath contained in a loosely stoppered Dewar flask. Temperature readings were obtained from a calibration curve for a copper-constantan thermocouple (2).

Pot heating was provided by a Variac-controlled heating mantle; the column and head heating coils were also controlled with variacs. The assembly for the fractionation included: pump, trap, manostat, ballast, manometer, and column. The manostat (Emil Greiner Co.) and ballast were included to minimize pressure fluctuations caused by changing atmospheric pressure.

The number of theoretical plates for the column, calculated from the separation effected with a liquid mixture, i.e., carbon tetrachloride and benzene, whose vapor and liquid compositions were fully known, was found to be 23.

Seven hundred cubic centimeters of the oil, which had been dried over anhydrous sodium sulfate for two weeks, was distilled at a 10/1 reflux ratio in this column. Eighty-eight separate fractions were collected, the first at 38–40°/4 mm. and the last at 89–90°/4.5 mm. Approximately 75 cc. of a black tarry residue remained in the pot at the end of the dis-

tillation. From preliminary physical and chemical tests it appeared that fractions 1–25, b. p. 38–41°/4 mm., consisted primarily of a relatively pure low boiling compound, hence these fractions were combined. Because of pressure fluctuations it was impossible to determine whether any of the higher boiling fractions consisted mainly of a pure component.

Distillation of the oil, using the Piros-Glover analytical column consigned to the University of Wisconsin by the Sinclair Refining Co., indicated the presence of one major component, b. p. 38–39°/3.2–3.6 mm., and a second component, b. p. 78–81°/2 mm.

Identification of 1,8-Cineole (Eucalyptol).—The presence of cineole was suspected in combined fractions 1–25 because of the characteristic odor and similarity of its physical constants and color tests to those of authentic cineole. Thermal analysis of these combined fractions indicated the presence of an appreciable amount of impurity. The cineole was removed and purified from these fractions as its *o*-cresol compound in the following way: A portion of the fraction was added to an excess of purified *o*-cresol at 50°, and the solution was allowed to cool to room temperature and stand for several hours. The fine white needles which formed were filtered, washed with cold petroleum ether, and dried. The derivative was decomposed with 5% aqueous NaOH, the aqueous layer removed, the oily residue taken up in ether, and the remaining *o*-cresol extracted from the ether solution with 5% NaOH. The ether solution was then washed to neutrality with water, dried over anhydrous Na_2SO_4 and the ether removed by distillation. The remaining cineole was distilled, b. p. 38°/4 mm., $n = 1.4605^{25°}$. The literature reports cineole, b. p. 38.9°/4 mm., $n = 1.454–1.461^{20°}$ (3, 4). Thermal analysis of this purified sample was carried out using a 10-junction copper-constantan thermocouple which had been calibrated previously against a strain-free platinum resistance thermometer, using freezing mixtures of inorganic compounds. A freezing point of +0.50° was observed for this sample; the f. p. of a pure authentic cineole was +0.75°. Figure 1 shows the time temperature curves of the purified cineole obtained from the oil and of a pure authentic cineole.

Further identification of cineole in combined fractions 1–25 was made by the preparation of several derivatives which are summarized in Table II. In each case tested there was no depression of melting point when the derivative was mixed with that prepared from authentic cineole.

Identification of *dl*-Carvone.—The addition of 2,4-dinitrophenylhydrazine reagent to several of the fractions from 62 to 88, b. p. 78–88°/4 mm., resulted in immediate clouding and the deposition of crystals within one-half hour; additional tests showed the presence of a ketone in these fractions. The ketonic material was removed from several of these fractions by means of Girard's Reagent "P" as follows (12): Approximately 5 Gm. of fraction in 10 cc. of alcohol containing 10% acetic acid was refluxed with 5.4 Gm. of Girard's Reagent "P" for one-and one-half hours. The mixture was cooled and poured into sufficient ice water (90 cc.) containing enough Na_2CO_3 (1.08 Gm.) to neutralize 90% of the acetic acid, so that the final concentration of alcohol did not exceed 10%. The solution was extracted with ether to remove nonketonic material and 10 cc. of conc. HCl was added. The solution was then allowed to stand at room temperature for twelve hours. The ketone was recovered by ether extraction, the ether extract dried and distilled to yield a light yellow oil, b. p. 87°/9 mm., $n^{25} = 1.4822$, $[\alpha]_D^{23} = +2.14°$. Identification of this material as *dl*-carvone was accomplished by the preparation of a number of derivatives which are summarized in Table III.

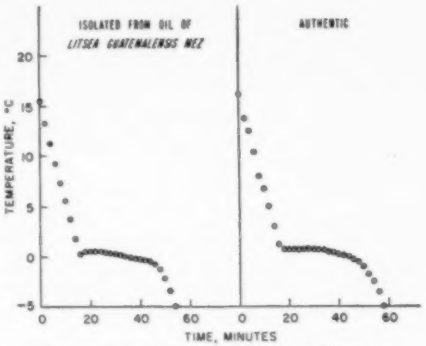

Fig. 1.—Thermal analysis of 1,8-cineole.

Linaloöl.—The nonketonic material remaining after isolation of *dl*-carvone from fractions 62–88 was investigated for presence of alcohols but no derivatives could be prepared. Chromic acid oxidation of this material gave a product reminiscent of citral, but it was insufficient for further study. Fractions 52–60, which were quite similar in physical properties, were combined, and a part of this was refluxed with alcoholic sodium hydroxide to saponify any esters. Duclaux values obtained from the acidified saponification mixture, after removal of the alcohol

TABLE II.—DERIVATIVES OF 1,8-CINEOLE

Derivative	M. P., ° C.	M. P. of Authentic Sample,[a] ° C.	Reported M. P., ° C.	References
o-Cresol	56–57	56–57	55.5	(5)
o-Chlorophenol	55–56	54–55	57–59	(6)
1,8-Cineolic acid	201–202.5	199.5–200	196–197	(7)
			204–206	(8)
Iodol	99–100	101–102	102	(9)
Resorcinol	78.5–83.5	.....	89	(10)
α-Naphthol	75.5–76	.....	73–75	(11)

[a] Prepared from Mallinckrodt Eucalyptol.

TABLE III.—DERIVATIVES OF *dl*-CARVONE

Derivative	M. P., ° C.	M. P. of Authentic Sample,[a] ° C.	Reported M. P., ° C.	References
Oxime	91–92.5		93–94	(13)
Semicarbazone	153–154	155.5–156.5	154–157	(14)
p-Nitrophenylhydrazone	177–178	179–180		
2,4-Dinitrophenylhydrazone[b]	185–186	186–187		

[a] Prepared by mixing equal amounts of *d*-carvone obtained from Caraway Oil and *l*-carvone obtained from Spearmint Oil.
[b] *Anal.*—Calcd. for $C_{16}H_{16}N_4O_6$: C, 58.17; H, 5.49; N, 16.96. Found: C, 58.32; H, 5.23; N, 16.80, 16.88.

and extraction with ether, were of no assistance in determining the possible presence of a volatile acid. Removal of the ether and distillation of the residue from the ether extract yielded a liquid, b. p. 73–74°/6 mm., $n^{25} = 1.4641$.

Although preliminary tests indicated this liquid to be an alcohol, no derivatives of it could be prepared. It was shown to be linaloöl by oxidation to citral and identification of the citral as follows: 5 Gm. of the liquid was shaken with Beckmann's chromic acid mixture and allowed to stand at room temperature for ten hours. The mixture was then extracted with ether, the ether solution washed with aqueous Na_2CO_3, dried and distilled to remove the ether. The oily residue was distilled, b. p. 85–87°/4 mm., $n^{24.6} = 1.4813$. The distillate had a strong lemon odor, reminiscent of citral. The β-naphthocinchoninic acid derivative was prepared as fine light yellow needles, m. p. 194–196°. Doebner (15) reports this derivative of citral, m. p. 197–200°; the same derivative, prepared from an authentic sample of citral, melted at 196–198°. Although geraniol is oxidized to citral under the same conditions, physical constants of the original fractions indicated the presence of linaloöl, predominately as the free alcohol rather than as the ester.

Hydrocarbon.—The oily residue remaining in ether solution, after removal of cineole as the *o*-cresol compound from fractions 1–25, was extracted with 5% aqueous NaOH to remove excess *o*-cresol, the ether solution dried and distilled to remove the ether. The residue was distilled under reduced pressure, b. p. 60–70°/9 mm., $n^{25.1} = 1.4715$, $\alpha^{25} = -14.85°$. This material was insoluble in syrupy phosphoric acid, indicating the presence of nonoxygenated compounds. It showed unsaturation, but attempts to prepare derivatives with bromine, HCl, nitrosyl chloride, and maleic anhydride were unsuccessful.

Analysis for Cineole.—Analysis of the original oil for its cineole content by the method of Cocking (5), based on the freezing point of the *o*-cresol addition compound, gave an average value of 49.6% cineole.

Analysis for Carbonyl Compounds.—Analysis of the original oil for its content of carbonyl compounds, calculated as *dl*-carvone, by the method of Hughes (16), using Girard's Reagent "T," gave an average value of 7.1%. However, this result must be regarded as questionable, since the small amount of precipitate obtained during the procedure was mixed with unreacted oil, resulting in a gummy residue, all of which could not be removed from the reaction flask. Drying at 110° resulted in blackening of the contents of the crucible.

SUMMARY

1,8-cineole and *dl*-carvone were isolated and identified in the volatile oil of *Litsea guatemalensis* Mez., and the presence of linaloöl was demonstrated. The presence of an unsaturated hydrocarbon was indicated but was not substantiated. The oil was shown to contain 49.6 per cent cineole.

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A Study of the Air-Blast Method for the Evaluation of the Effectiveness of Flavonoid Compounds on Capillary Fragility*,†

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Evaluation of the potency of flavonoid compounds has been determined by an air-blast method which is described. The method used appeared to indicate that the drugs tested protected the lungs of mice from extensive capillary rupture, but actually is of slight value because the results obtained were in general not statistically significant.

THE reports by Griffith, *et al.* (1), and Shanno (2) that rutin, a flavonoid compound, increased capillary resistance led to a search for more potent compounds. Many methods have been proposed for a biological assay, potentiation of epinephrine (3–10), capillary damage in scorbutic guinea pigs (11, 12), dye diffusion into the tissues (13), and capillary rupture in the lungs of mice subjected to a rapid decrease in atmospheric pressure (14, 15). Only the latter can be said to measure capillary strength or resistance, but it has been shown that this method is subject to considerable error (16).

In blast damage extensive capillary rupture in the lungs is evident and it seemed that under rigidly controlled conditions the extent of damage could be easily reproducible from animal to animal in a given group. Such reproducibility of air-blast damage has been demonstrated by Cassen, *et al.* (17), using mice. This method offered an excellent means for testing the ability of the flavonoid compounds to decrease capillary fragility because under the conditions employed other vascular factors such as permeability would not influence the results.

It has been postulated that the air pressure wave upon contact with the body of the animal is transmitted as a fluid shock wave in the tissues and body fluids. Upon coming in contact with an air interface, as in the lungs, a decrease in resistance is encountered. This results in the reflection of a tension wave which causes rupture of the capillary and alveolar membranes and the production of varying degrees of hemorrhage. Both external and internal pressure waves produce such damage.

EXPERIMENTAL

Male albino mice weighing 10 to 31 Gm. (average 18 Gm.) were attached to the recoil loading mechanism by clamping the four limbs near each foot. See Fig. 1 for the entire apparatus. This placed the animal in a position so that the air-blast wave hit it head-on, thus causing considerable damage to the lungs. The loaded recoil mechanism was placed in the steel blast tube, the pressure chamber filled with air to a pressure which would result in a blast wave of 18 lb./sq. in., and the cellulose acetate membrane was ruptured by means of a three-bladed knife. The mouse was then removed from the tube and sacrificed, using a sudden knife stab to cut the aorta, preventing additional ecchymoses in the lungs due to agonal engorgement. This was done because preliminary studies in which the animals were sacrificed with barbiturates, volatile anesthetics, or decapitation confirmed the findings of Maitland, *et al.* (18), that ecchymotic areas were produced in the lungs of normal animals unless they were sacrificed by snipping the aorta. The lungs were removed and the hemorrhage score evaluated arbitrarily according to the scheme shown in Fig. 2. Although this method may appear complicated, it was readily mastered and reproducible. The standard curve, Fig. 3, was determined with normal male and female mice, a total of 590 mice having been used. The population standard deviation, 2.3 hemorrhage score units at 18 lb./sq. in., was determined with 221 animals. However, the standard deviation, σ 20 shown on Fig. 3 is estimated for the average of the scores of groups of 20 animals each by taking the population standard deviation ($\sigma = 2.3$) and dividing it by the $\sqrt{20}$. Therefore, at 18 lb./sq. in. we obtain a value of 4.8 ± 0.5 hemorrhage score units.

The flavonoid compounds evaluated were dissolved in distilled water to which was added a very small amount of 1 *N* NaOH. No heat was employed in order to prevent the formation of chalcones. The *p*H values of these solutions were determined with a Beckman Model G *p*H meter and were found to be in the range of 11.82 to 12.1. Control animals were injected with the same volume of physiological saline as used in the medicated animals. As there might be a variation in the mice used, an equal group of normal control animals was always used in each drug evaluation. Further-

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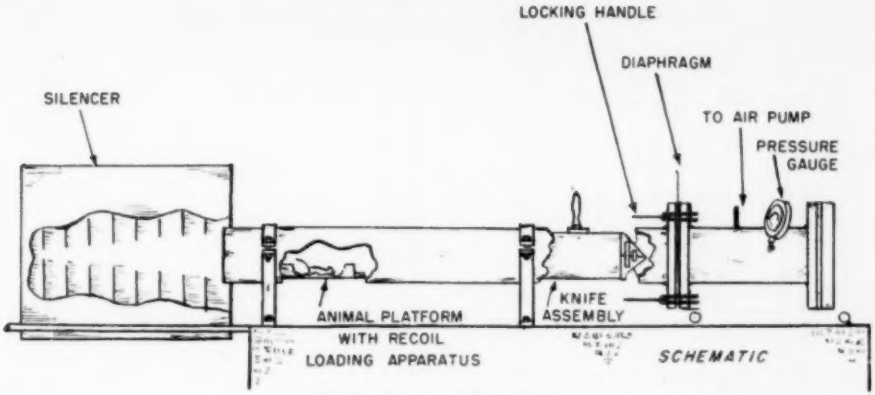
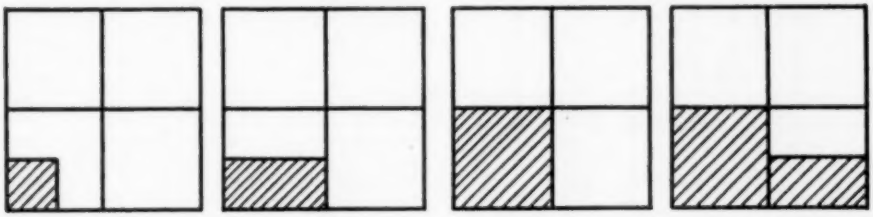


Fig. 1.—Blast tube assembly.

more, when it appeared that the results obtained might be due to the solvent used, as has been previously reported (15, 16), a group of animals was injected with physiological saline containing the amount of 1 *N* NaOH required to solubilize the drugs. Also, to determine the type of evaluation that could be obtained when the results were based upon a value greater than the LD_{50} blast pressure, a group of animals was subjected to a blast pressure of 20 lb./sq. in. In all the medicated animals, the drugs were given intraperitoneally, and the animals were not blasted until one hour later in order that the greatest amount of drug absorption could take place. The fact that the urine had the orange-red color usually observed after flavonoid administration was evidence that the drugs were being absorbed.

RESULTS

The lung hemorrhage scores for the different flavonoids at varying doses are given in Table I. Inspection of these scores indicates a decreased lung hemorrhage score after flavonoid administration.

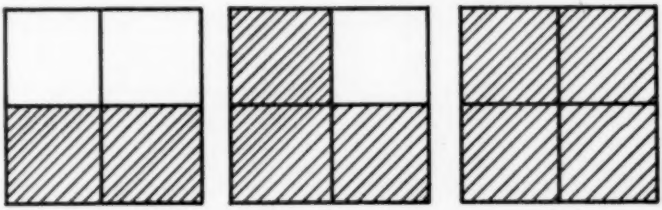


1
Less than one-eighth of the lobe involved, and more than petechiae

1¼
one-eighth to one-fourth of the lobe involved

1½
one-fourth to three-eighths of the lobe involved

1¾
three-eighths to one-half of the lobe involved



2
one-half of the lobe involved

3
three-fourths of the lobe involved

4
all of the lobe involved

Fig. 2.—Numerical evaluation of lung damage. Each large square represents one lobe. The shaded areas are graphic representations of the numerical values assigned to the hemorrhagic areas caused by the blast. Petechiae, blemishes, slight discolorations, etc., are not reported. The lung damage for each of the five lobes is added to give the total lung damage. The total lung damages are added and divided by the number of mice per test to give the average lung damage.

TABLE I.—NUMERICAL EVALUATION OF LUNG DAMAGE DUE TO 18 LB./SQ. IN. BLAST PRESSURE USING 20 MICE PER DOSE

Dose, (Mg./Kg.)	Hesperidin		Hesperidin Methyl Chalcone		Phosphorylated Hesperidin		Homoeriodictyol		Naringin		Rutin		Rhamnetin		Quercetin	
	LD8	D	LD8	D	LD8	D	LD8	D	LD8	D	LD8	D	LD8	D	LD8	D
100	4.89	1.04	4.96	2.26	4.80	3.14	3.71	1.18	6.65	−0.60	5.79	−0.30	4.70	2.96	5.56	1.16
75	4.55	1.72	5.34	1.50	5.13	2.48	4.80	−1.00	5.19	1.12	4.93	1.42	5.27	2.02	4.60	3.08
50	4.96	0.90	5.55	1.80	5.25	2.24	4.82	−1.04	5.38	0.74	5.58	0.12	5.08	2.20	4.64	3.00
25	5.20	0.42	5.57	1.04	6.43	−0.12	4.34	−0.08	5.28	0.94	5.40	0.48	6.06	0.24	5.53	1.22
0																
(Control; saline + 1 N NaOH)	5.41	...	6.09	...	6.37	...	4.30	...	5.75	...	5.64	...	6.18	...	3.19	...
No injection	...	...	...	...	...	...	...	...	...	...	...	...	...	...	6.14	...

LD8 signifies lung damage score according to Fig. 2.
D signifies the difference between 2(LD8) for each dose and 2(LD8) for the control obtained in Standard Deviation Units according to: $LD8/\sigma 20$ where the standard deviation in hemorrhage score units is 0.5.

Fig. 3.—Lung damage vs. shock pressure. —— Smoothed average damage curve. - - - Smoothed standard deviation limits for averages of groups of 20 animals. ● Experimentally determined values.

This is particularly evident when the scores of the controls are compared with those obtained with the higher dosage levels of the drugs. However, it is apparent that with naringin, rutin, and quercetin doses of 100 mg./Kg. the lung capillary fragility was increased rather than decreased. Furthermore, a definite abnormal alkaline saline control value is evident in the quercetin series. This can be corrected if the noninjected control value is used. The results thus obtained will be in general agreement with the values seen with the other compounds. The use of sodium hydroxide as a solubilizing agent was found to be undesirable because when two groups of mice, one injected and one noninjected, were blasted at 18 lb./sq. in., the values obtained were 5.39 and 7.13, respectively. This would indicate that alkali was beneficial in reducing total lung hemorrhage, whereas the values are not statistically significant. In this regard, the only values given in Table I which can be considered significant, and thus indicate a trend in the direction of an increased capillary resistance, are those in column D which have a value of 3 or greater. Such values occurred only with phosphorylated hesperidin, rhamnetin, and quercetin.

Table II presents the effect of rutin upon the lethality of 20 lb./sq. in. blast pressure. It may be noted that there is a marked day-to-day variation in protectant effect of rutin, while there is little change in mortality ratios of both control groups. However, when the number of animals used in each group is expressed as a total mortality ratio the values obtained indicate that rutin has only a very slight protectant effect.

TABLE II.—EFFECT OF RUTIN ON THE 24-HOUR MORTALITY RATIO OF MICE SUBJECTED TO AN AIR BLAST PRESSURE OF 20 LB./SQ. IN.

Drug	pH	No. of Animals	Mortality Ratio		
			First Day	Second Day	Total
Rutin, 50 mg./Kg	11.82	35	2/18	7/17	9/35
Alkaline saline control	11.88	35	5/18	6/17	11/35
Uninjected control	...	35	7/18	6/17	13/35

DISCUSSION

It appears from our results, as well as those of previous investigators (14–16), that regardless of the means used, external or internal air pressure, the method of lung capillary rupture is of little value in the determination of flavonoid potency. Perhaps these results are due to the lung capillaries being relatively short, 0.5 to 1 mm. long, and their not being surrounded by large masses of supportive tissue as in the skin where the usual petechial index type of evaluation is performed. It may be, however, that these drugs exert their effects by modifying capillary permeability, and thus any attempt to evaluate them for their ability to prevent capillary rupture is erroneous.

The need for starving the animals prior to testing was reported by Kibrick and Goldfarb (16) to be one of the variables which should be rigidly controlled for the attainment of accurate results. However, Raiman and Necheles (15) found no great difference in the results obtained with fed or starved mice. We used animals which had been fed, and our results were similar to those reported by them and by Majovski, *et al.* (14). Furthermore, when a comparison is made of the number of animals per group employed by us and the number employed by previous investigators (14–16), there can be little doubt that feeding has no real effect upon the results obtained.

SUMMARY

A study has been made of an air blast method for the evaluation of the potency of flavonoid compounds. The method, while it seemed to show that these drugs protected the lungs of mice from extensive capillary rupture, was of little value because the results obtained were in most cases not statistically significant. The use of NaOH as a solubilizing agent was found to be detrimental because that chemical exerted some slight protectant action itself.

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Microtoxicology VII. Identification and Differentiation of the Antihistaminics of the Thiodiphenylamine Series*

By THOMAS J. HALEY†,‡ and GEORGE L. KEENAN§

The results of a study of the application of colorimetric and precipitation reactions to six antihistamine drugs of the thiodiphenylamine series are reported. Optical crystallographic properties of the drugs have been determined and data on these properties are presented. Buckingham's reagent has been found to be applicable for identification and differentiation of the drugs studied. Optical crystallographic methods are best for identifying Phenergan and Pyrrolazote.

HALPERN and his co-workers (1–8) have recently reported upon the antihistaminic properties of a series of compounds derived from thiodiphenylamine. Several other investigators (9–11) have found that two drugs of this series 3015 RP or SC 1627 (N-dimethylaminoethyl thiodiphenylamine hydrochloride) and Phenergan, 3277 RP (N-dimethylamino isopropyl thiodiphenylamine hydrochloride) are more active than some of the antihistaminics in current use. Recently, Vander Brook, *et al.* (12), reported that Pyrrolazote (*Beta*-pyrrolidine-ethyl-thiodiphenylamine hydrochloride) was also a very potent antihistaminic.

In order to complete the identification and differentiation of all of the antihistaminics, we decided to subject the available drugs of this series to the same tests used to identify the other antihistaminics (13–16). Furthermore, to prevent confusion and misinterpretation, the results obtained are compared with those recorded in the literature for drugs other than the antihistaminics.

EXPERIMENTAL

The compounds investigated besides those named above were SC 1742 (N-trimethylaminoethyl thiodiphenylamine chloride), SC 1898 (N-dimethylethanolaminoethyl thiodiphenylamine bromide), and SC 1923 (N-methylethanolaminoethyl thiodiphenylamine hydrochloride). The reagents used were prepared as directed by Thienes and Haley (18). To one drop of reagent on a microscope slide, or in the well of a spot plate, was added about 1 mg. of the compound to be tested and the resulting precipitate or color observed for thirty minutes. All crystalline precipitates produced were examined for their crystallographic characteristics by the immersion method. The end point with the acidic colorimetric reagents was a charring or the development of a stable color. Where charring occurred, only the colors produced prior to this were considered. The results obtained with both types of reagents are given in Table I. Table II gives the uncorrected melting points of the antihistaminics as determined by use of the Thiele tube. To further aid in the identification of these antihistaminic drugs, photomicrographs of their pure crystals were taken with a Visicam photographic attachment on a B and L binocular microscope using the 16-mm. objective and the 10x eyepiece. See Fig. 1. Inasmuch as the precipitation and colorimetric tests were almost identical for each compound, the optical crystallographic properties of the pure compounds were studied. 3015 RP, SC 1742, SC 1898, and SC 1923 could not be examined crystallographically due either to the minute size of the particles or to unsatisfactory orientation. This is illustrated in the photomicrographs.

Fig. 1.—Phenergan, 3277 RP (N-dimethylamino isopropyl thiodiphenylamine hydrochloride).

PHENERGAN HYDROCHLORIDE

In Ordinary Light.—Colorless rods and irregular fragments describe the habit of the substance.

Characters Shown in Parallel Polarized Light (Crossed Nicols).—The extinction is parallel and the sign of elongation positive.

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TABLE I.—COLORIMETRIC AND PRECIPITATION REACTIONS OF THIODIPHENYLAMINE ANTIHISTAMINICS

Reagent	SC 1627, 3015 RP	SC 1742	SC 1898	SC 1923	Phenergan 3277 RP	Pyrrolazote
Conc. H_2SO_4	Pink	Pink	Magenta	Pink	Fuschia	Magenta
Conc. HNO_2	Magenta changing immediately to orange-yellow	Brilliant red changing immediately to orange-yellow	Magenta changing immediately to orange-yellow	Magenta changing immediately to orange-yellow	Magenta changing immediately to orange-yellow	Magenta changing immediately to greenish yellow
Mandelin's	Pink	Pink	Pink	Pink	Pink	Pink
Marquis'	Magenta	Magenta	Magenta	Magenta	Magenta	Magenta
Buckingham's	Deep red	Chocolate-brown	Reddish brown	Brownish black	Brilliant red changing to magenta	Magenta, then brown, finally black
Frohde's	Pink	Pink	Magenta	Pink	Pink	Magenta changing to orange-red, greenish edges
Chloroplatinic acid	Black amorphous mass. Reagent turns blue-green	Black amorphous mass. Reagent turns blue-green	Black amorphous mass. Reagent turns blue-green	Black amorphous mass. Reagent turns blue-green	Flat purple leaflike crystals, single or in crosses. Reagent turns blue-green	Purple pointed rods. Reagent turns blue-green
Picric acid, sat. aq. soln.	Amorphous	Amorphous	Amorphous	Amorphous	Amorphous	Amorphous
Bromine water, 1%	Red immediately fading to colorless	Red immediately fading to colorless	Red immediately fading to colorless	Red immediately fading to colorless	Red immediately fading to colorless	Red immediately fading to colorless

Characters Shown in Convergent Polarized Light (Crossed Nicols).—Only flash interference figures observed. Optic sign apparently (—).

Refractive Indices.—$\alpha = 1.617$, $\beta = 1.691$, $\gamma = 1.733$; all ± 0.002.

PYRROLAZOTE HYDROCHLORIDE

In Ordinary Light.—The substance consists of colorless, stout, prismatic forms readily breaking up into irregular fragments for microscopic examination.

In Parallel Polarized Light (Crossed Nicols).—The fragments invariably extinguish sharply with crossed nicols and many of them exhibit anomalous interference colors consisting of peculiar shades of blue and purple.

In Convergent Polarized Light.—No interference figures observed.

Refractive Indices.—The substance is characterized by having very high refractive indices, the lowest measurable by the immersion method being 1.690 ($\pm$ 0.003). The maximum value (γ) closely approximates that of methylene iodide (1.737). The β-value could not be determined on the substance.

TABLE II.—MELTING POINTS OF THIODIPHENYLAMINE ANTIHISTAMINICS

Drug Name or Number	Chemical Name	M. P.	B. P. Free Base	Reference
3015 RP, SC 1627	N-dimethylaminoethyl thiodiphenylamine HCl	201–201.5 212[a]	183–187	19 ..
SC 1742	N-trimethylaminoethyl thiodiphenylamine chloride	219–221[a]	...	..
SC 1898	N - dimethylethanolaminoethyl thiodiphenylamine bromide	147–148[a]	...	..
SC 1923	N-methylethanolaminoethyl thiodiphenylamine HCl	176–177[a]	...[a]	..
Phenergan 3277 RP	N - dimethylaminoisopropyl thiodiphenylamine HCl	204 178–181[a]	190–192 ...	19 ..
Pyrrolazote	β-pyrrolidine-ethyl thiodiphenylamine HCl	196–197 197–198[a]	... ...	20 ..

[a] Signifies uncorrected melting point.

DISCUSSION

The reactions of the thiodiphenylamine antihistaminics with concentrated sulfuric acid are similar to those reported for Neo-Antergan, 929 F (16), Bromothen, Chlorothen, and Tagathen (15). However, the initial color of 929 F and the final colors of the latter three compounds make differentiation very easy. No other alkaloids give colors resembling those described above.

Fig. 2.—Pyrrolazote (*Beta*-pyrrolidine-ethyl-thiodiphenylamine hydrochloride).

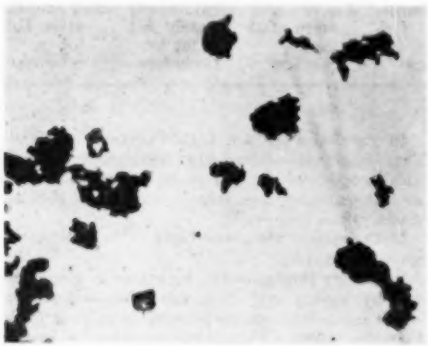

Fig. 3.—SC 1742 (N-trimethylaminoethyl thiodiphenylamine chloride).

Fig. 4.—SC 1898 (N-dimethylethanolaminoethyl thiodiphenylamine bromide).

The initial color obtained when SC 1742 is mixed with nitric acid enables a differentiation to be made between this compound and the other drugs of this series. However, the color change produced is identical with that given by brucine and similar to that seen with morphine (17). The final color produced with reagent, while it prevents confusing these antihistaminics with the others previously reported (14–16), makes it difficult to differentiate them from codeine, narceine, narcotine, papaverine, physostigmine, strychnine, thebaine, or veratrine (17).

Both Mandelin's and Marquis' reagents give the colors which are useless for differentiating these drugs from each other. Furthermore, unless other tests are employed, the thiodiphenylamine derivatives may be mistaken for either Neohetramine or Neo-Antergan (16).

Buckingham's reagent is the best one for differentiating these drugs from each other and when combined with the other tests given in Table I allows a positive identification to be made. Brucine gives a color similar to 3015 RP but the two compounds react differently to the other reagents used so that no confusion should result.

Frohde's reagent is very useful in adding confirmatory evidence in the identification of Pyrrolazote because the color sequence differs from those reported for the other antihistaminics (14-16) and

Fig. 5.—3015 RP or SC 1627 (N-dimethylaminoethyl thiodiphenylamine hydrochloride).

alkaloids (17). The reactions of the other thiodiphenylamine derivatives, while differing from the alkaloids (17) and antihistaminics (14–16), are of little use for differentiation.

The bromine water test apparently identifies the thiodiphenylamine portion of the molecule because the parent compound, phenothiazine, gives an identical color reaction differing only in greater stability of the color produced (21).

The precipitates produced by chloroplatinic and chlorauric acids were of little value because where crystals were produced they were such that no optical crystallographic study could be made. However, the color reactions of these reagents are valuable because the other antihistaminics do not give such color tests (14–16).

As with most of the other antihistaminics investigated, the picric acid reagent produced only amorphous precipitates and is therefore of little value.

The discrepancies in the melting point values reported for both 3015 RP and Phenergan cannot be explained. In the case of Phenergan, we tested a sample produced in France as well as one produced in this country and found their melting points in agreement. Furthermore, our results with Pyrrolazote are within the range reported in the literature so that the apparatus and method used cannot be at fault.

SUMMARY

Microchemical data covering colorimetric and precipitation reactions and optical crystallographic properties of six antihistaminics of the thiodiphenylamine series have been presented. Buckingham's reagent is the best colorimetric one for identifying and differentiating these drugs. The best means for identifying Phenergan and Pyrrolazote is by optical crystallographic methods.

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Microcrystallographic Descriptions of Some Crystalline Anticonvulsants*,†

By GEORGE L. KEENAN‡

Microcrystallographic characteristics of diphenylhydantoin sodium (Dilantin Sodium), 3 - methyl - 6,5 - phenyl - ethylhydantoin (Mesantoin), and phenobarbital have been determined and are reported. The microcrystallographic data recorded are useful in establishing the identity of the three compounds either separately or in combination.

THE THREE anticonvulsants, Dilantin Sodium, Mesantoin, and phenobarbital, used in combination in the treatment of epilepsy, have been found to reveal distinctive microscopic features that are of analytical interest in establishing their identity.

Chemically, Dilantin Sodium and Mesantoin are both derivatives of the condensation product of urea and acetic acid, while phenobarbital is a derivative of the condensation product of urea and malonic acid. These three substances are crystalline and well adapted for microscopic study, with the possible exception of Dilantin Sodium. The latter is somewhat hygroscopic and gradually absorbs carbon dioxide when exposed to the air, thereby liberating diphenylhydantoin. This behavior precludes a microscopic study of the substance *per se*, but, as will be shown later, diphenylhydantoin may be readily prepared from it and the optical constants of the latter determined.

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EXPERIMENTAL

This microscopic study includes general observations and those readily revealed with the polarizing microscope. Principal emphasis is placed upon significant features that can be observed in ordinary light, parallel polarized light (crossed nicols), and the conoscopic examination, when applicable. The description for each substance first will be given, followed by a summation of the distinctive features of the three compounds. (For brevity, the shorter names of the substances will be used in these descriptions.)

Mesantoin.—$C_{11}H_{14}N_2O_2$. *In ordinary light* the habit is characterized as thin, colorless rhombs and some six-sided forms, also plates tipped on edge, in this orientation suggesting fibrous fragments. *In parallel polarized light (crossed nicols)*, the thin plates show low-order whites. On the elongated plates, the extinction is parallel and the sign of elongation positive. Many of the thin plates do not

extinguish sharply with crossed nicols. *In convergent polarized light (crossed nicols)* the thin plates not extinguishing sharply show a biaxial interference figure with the acute bisectrix and negative optic sign. 2E is small (50 ± 5° obs.). *Refractive indices:* $\alpha = 1.570$, $\beta = 1.600$, $\gamma = 1.605$, all ±0.002. β and γ can be measured on plates showing the plane of the optic axes and with negative optic sign. α and γ are frequently shown on plates tipped on edge (having the appearance of small fibers).

Mesantoin readily crystallizes from 95% alcohol and water on an object slide. To a very small amount of the material on the slide, a drop of 95% alcohol is added. The substance quickly dissolves and then a drop of water is introduced. Short, numerous, thin rhombs will crystallize out. To hasten the crystallization, the test drop can be reseeded by stirring. Under these conditions, these rhombs will also quickly form from powdered tablet material (containing starch) as well as from the undiluted substance.

Diagnostic Microscopic Features.—The following are the outstanding microscopic characters of Mesantoin:

1. The habit, consisting of thin, colorless rhombs, readily formed from 95% alcohol and water.
2. The frequent occurrence of plates tipped on edge and resembling small fibers (similar to talc in this respect).
3. The low-order whites shown on plates when examined with crossed nicols.
4. The acute bisectrix interference figures showing small axial angle with negative optic sign.
5. The close similarity of the β and γ refractive index values. If the powdered substance is immersed in either the β or γ liquid and observed in ordinary light, the margins of the plates will have essentially disappeared on the greater proportion of the crystals. In fact, in tablet material, only the starch grains will be visible, but upon crossing the nicols, the thin, colorless plates will be observed.

Dilantin Sodium.—$C_{15}H_{11}N_2O_2Na$. For reasons already given, the monosodium salt of 5,5-diphenylhydantoin is not suitable for microscopic examination by the immersion method. However, for the purpose of characterizing the substance microscopically, it is convenient to dissolve a small amount in a drop of 95% alcohol on an object slide, then adding a drop of 5% hydrochloric acid. Rodlike plates and fine needles of diphenylhydantoin separate out, readily dry on the slide at room temperature and may be subsequently examined with the polarizing microscope. *In parallel polarized light (crossed nicols)* the extinction is parallel and the sign of elongation negative. Low-order whites are shown on the very thin plates. *In convergent polarized light (crossed nicols)* no interference figures are observed. *Refractive indices:* $\alpha = 1.600$ (commonly shown lengthwise), $\gamma = 1.635$ (commonly shown crosswise); both ±0.002. β was not determined due to lack of interference figures.

Diagnostic Microscopic Features.—The most significant microscopic characters of Dilantin Sodium are:

1. The ease with which crystalline diphenylhydantoin may be prepared from it on the slide by the addition of 95% alcohol and 5% hydrochloric acid, resulting in the formation of colorless rodlike plates and needles.
2. The frequent occurrence of the minimum and maximum refractive index values of diphenylhydantoin in the orientations indicated.

Phenobarbital.—$C_{12}H_{12}N_2O_3$. *In ordinary light* the substance consists largely of colorless rhombs, frequently elongated, and six-sided forms. *In parallel polarized light (crossed nicols)* the extinction is parallel on the elongated forms and sign of elongation negative. Many of the plates do not extinguish sharply. *In convergent polarized light (crossed nicols)* partial biaxial interference figures are observed with negative optic sign. *Refractive indices:* $\alpha = 1.557$, $\beta = 1.620$, $\gamma = 1.667$, all ±0.002. The α and β values are most commonly found.

TABLE I.—SUMMARY OF MICROSCOPIC CHARACTERS

	Mesantoin	Dilantin Na[a]	Phenobarbital
Habit	Rhombs	Rods, needles	Rhombs, parallelograms
Extinction	Parallel	Parallel	Parallel
Elongation	Positive	Negative	Negative
Class	Biaxial	Biaxial	Biaxial
Sign	Negative	?	Negative
2E	50 ± 5°	?	?
Indices			
α	1.570	1.600	1.557
β	1.600	?	1.620
γ	1.605	1.635	1.667

[a] These data refer to diphenylhydantoin prepared as described from Dilantin Sodium.

To facilitate the orientation of the crystals in respect to the most commonly occurring refractive indices, it is convenient to dissolve a small amount of phenobarbital in a drop of 95% alcohol on an object slide, followed by a drop of water. Soon the edge of the drop will become "milky" in appearance and eventually a copious crop of parallelograms will come out. The preparation may be allowed to dry at room temperature and examination subsequently made with the polarizing microscope. In this condition, the parallelograms usually show a refractive index of 1.557 (α) lengthwise and 1.620 (β) crosswise. Plates tipped on edge will show 1.557 (α) lengthwise and 1.667 (γ) crosswise.

Diagnostic Microscopic Features.—Phenobarbital exhibits the following significant features microscopically:

1. The significant habit of parallelograms when crystallized from 95% alcohol and water.
2. The frequent occurrence of the α and β index values, shown respectively, lengthwise and crosswise.
3. The frequent occurrence of the α and γ index values on plates tipped on edge.

The Precision of the Line Test for Assay of Vitamin D in Fish Oils*

By C. A. MORRELL†,‡ and E. M. ORDWAY†,§

The results of an examination of the precision of the line test for determining the vitamin D potency of fish oils is presented. The data were obtained from routine tests and comparisons of the International Standard for vitamin D carried out at the same time. Variability in results due to assessment of the degree of healing of bones by different observers was studied. The precision obtained in the assay as conducted in this laboratory is somewhat less than that estimated by the British Pharmacopeia or obtained by British workers. For economic reasons, the small increase in precision obtained by combining the estimated values of several observers for healing of bones does not appear to justify the requirement that degrees of healing of rickets be assessed by more than one worker.

THERE ARE many factors which tend to decrease the precision of the line test for vitamin D. An estimate of the precision possible by British workers is indicated in a recent publication (1).

During routine tests of samples of fish oils a few years ago in this laboratory a study was made of the estimated and actual precision obtained for samples and the International Standard preparation for vitamin D.

One of the variable factors affecting the precision of the test is the reading or value assigned to the line of healing for rickets in the rat bones. Although a standard set of pictures such as that prepared by Coward (2) is used, nevertheless the value assigned to any particular bone is a matter of opinion within certain limits. In our experience there are some observers who estimate the values of a group consistently higher or lower than others. If a standard of comparison, such as the International Standard, is used these differences tend to cancel themselves since the bones from rats fed the standard are also evaluated higher or lower as the case may be. It was thought desirable, however, to compare the results obtained by individuals with each other and also to learn if the precision was improved by averaging the results of a number of observers and using the average readings to calculate the relative potency.

In addition, an attempt was made to determine the accuracy possible by use of the test under known and favorable conditions. For this purpose a number of assays were included in which the International Standard was compared with itself. By so doing a material of known potency was used and any divergence from the known value can be considered an error in the method under the conditions described.

METHODS

The Line Test.—Wistar albino rats bred in our colony from mothers on a diet low in vitamin D were used for the test. They were weaned at twenty-eight days of age and fed a rachitogenic diet made up as follows:

Diet 42A—D

Ingredient	%
Sodium chloride	1
Calcium carbonate	6
Brewer's yeast	3.3
Wheat gluten	19.7
Ground corn	70
Distilled water	*ad libitum*

After twenty-eight days on this diet the animals were divided into groups of 10, the same number of rats from each litter being included in each of the groups as far as their numbers would permit. Each group contained the same number of rats of each sex; usually there were 5 males and 5 females in each group. Rats were weighed at the beginning and end of the test period. Males weighing between 40 and 55 Gm. and females between 38 and 50 Gm. were used. Each test included a control group of at least 10 rats which were not fed vitamin D. During the test the rats were kept in a room free from daylight. The doses of 0.1 cc. were administered *per os* by clean dry luer tuberculin 1-cc. syringes and shortened 18-gauge needles covered with catheter tubing. A separate needle and syringe were used for each solution. The doses of vitamin D were administered every afternoon for six consecutive days and two additional days were allowed without vitamin D at the end of the feeding period. After that time the rats were sacrificed with coal gas and the bones of the right foreleg dissected out and dropped into 4% formalin solution. After a few days to two weeks in this solution the radius and ulna were separated and sectioned longitudinally by means of a razor blade. The four bone sections

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† Our thanks are due to Dr. L. I. Pugsley and to J. W. Sinclair for independently evaluating the degrees of healing of rickets in all of the tests reported.
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§ Technical Assistant to Director.

from each rat were placed for a few minutes in a small glass cup containing a solution of 1.5% silver nitrate. The sections were then washed with distilled water and exposed to light. The degree of healing was recorded following the chart given by Coward (2).

It is important to note that each rat was numbered serially and the bones given a code number. The cups containing the bone sections were arranged in a random fashion so that observers were not aware of which dose or test material was related to any particular bone. Four experienced observers independently read each section and the four readings from each cup were recorded separately opposite the given code number.

For calculating the results obtained by each observer the four readings for each bone were averaged and finally the mean for the group determined from the ten individual averages. For calculating the combined average reading of the degree of healing in each rat, the 16 readings, four by each of four observers, were averaged. Group means were obtained from the ten averages so calculated.

Following the above procedure it is believed that bias in readings is eliminated.

The control groups which received no vitamin D were included in the above scheme and were separated from the test rats by use of the code numbers. Although the average reading on the Coward scale for control was about 0.16, the large majority of control rats showed no healing.

Test Solutions.—The materials assayed in this series of tests were the International Standard and commercial preparations obtained by food and drug inspectors from the retail trade and the manufacturers for one reason or another. The labeled potency of the commercial preparations was assumed correct for dilution purposes. The dilutions of the International Standard were made to yield a strength for dosing purposes of about 0.8, 0.4, 0.2 International Unit in 0.1 cc. of the high, middle, and low doses, respectively. This potency was found by experience to produce satisfactory degrees of healing in rats of our colony raised and fed as described.

The doses were weighed on a balance sensitive to 0.1 mg. and transferred to 100-cc. graduated glass-stoppered cylinders by washing with corn oil and diluted to volume with corn oil. This solution represents the high dose in each case. A portion was diluted to twice its volume with corn oil for the middle dose, thoroughly mixed, and a portion of this was similarly diluted in turn for the low dose. The solutions were thoroughly mixed when made up to volume and were kept in the refrigerator until two hours before use, at which time they were brought to room temperature.

Calculation of Results.—The method of computation described by Irwin (3) was used throughout.

RESULTS

Table I gives the results of 27 assays in which the International Standard for vitamin D was assayed in terms of itself. As described in the previous section on methods, three dilutions of the standard were made from one weighing of the oil. Each of these three solutions was divided into two parts, properly marked, and one set was fed as the International Standard and the other fed as the International Standard Repeat, the latter being treated as an unknown or test sample. Obviously differences in results between the International Standard and the International Standard Repeat could only be due to errors in the method of test.

The results in Table I show the determined potency of the International Standard as the repeat sample in terms of International Units per gram. Obviously they should all be 1000 I. U. per gram if the method had perfect accuracy. The differences observed are due to errors of the method.

The average of these twenty-seven comparisons is 1014 International Units and the standard deviation of a result is 18.7% of the average, i.e., 66% of the results of a large series of such tests should fall between 995 and 1033 International Units.

Figure 1 shows in graphic form a frequency distribution of the twenty-seven determinations of the International Standard as reported in Table I. The distribution is somewhat asymmetric as predicted by the estimated confidence limits.

The evaluation of the results of the test as indicated by the thickness, character, and continuity

TABLE I.—ACCURACY AND PRECISION OF VITAMIN D ASSAYS; COMPARISON OF SOLUTIONS OF INTERNATIONAL STANDARD OF VITAMIN D[a]

Assay No.	Determined Potency, I. U./Gm.	Confidence Limits as % of Determined Potency	Assay No.	Determined potency, I. U./Gm.	Confidence Limits as % of Determined Potency
51	796	75–133	65	879	71–141
52	711	62–161	73	1091	74–135
53	1224	63–158	74	1025	71–141
54	1137	64–156	75	1045	72–139
55	948	57–175	76	1003	70–143
56	1030	61–164	77	796	71–141
57	785	63–158	78	996	68–147
57-b	779	70–143	79	1302	51–196
59	822	62–161	80	1119	69–145
60	849	57–175	82	1575	66–151
61	1150	68–147	84	965	64–156
62	1008	54–185	84-b	1044	66–151
63	1048	57–175	84-c	986	66–151
64	1255	62–161			

[a] True potency 1000 I. U. per Gm. Mean and standard deviation of determined potencies 1014 ± 190 I. U. per Gm.

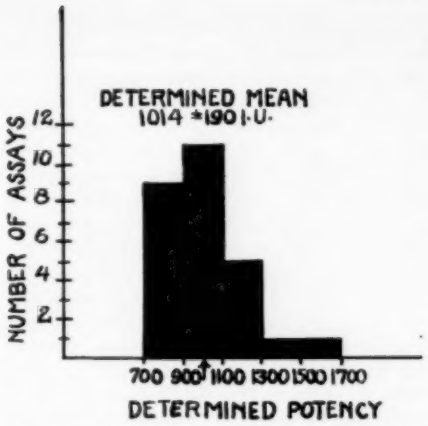

Fig. 1.—Graphic presentation of frequency distribution of the 27 determinations of the International Standard as reported in Table I.

of the line of healing depends on the opinion of the observer as to whether the line resembles more closely one or another of a selected series of standard pictures such as that of Coward (2). Because of this, the results are subject to errors of judgment which vary in degree with the individual observer and consequently it is of interest to know whether some observers are more consistent in their judgment than others. Two factors may be considered in this variation: (*a*) whether any particular individual was consistently and significantly further from the truth as evidenced by the size of the difference of his assay results from the true value; or (*b*) whether any particular indivitual was less consistent in reading bones with the same degree of healing. This latter would lead to less precise values being obtained and would be evidenced by larger confidence limits or greater estimated "limits of error."

A study of the first of these factors is presented in Tables II and III in which analysis of variance has been employed. The figures in the body of Table II are the differences between 1000 International Units (the true value of the oil) and the determined value for each of four observers calculated separately and the combined results of the four observers in the column marked "C." Ten assays of the International Standard reported in Table I were used for the laborious calculations. Table III gives the analysis of variance for the figures in Table II. There is no indication that there is a significant difference among the figures in the five columns of Table II. This may be interpreted as indicating that there is no significant gain in accuracy by combining the readings of bones by four observers and that there is no significant difference in the results obtained by the four observers.

TABLE III.—ANALYSIS OF VARIANCE OF FIGURES IN TABLE II

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square	*F*
Total	49	476,819		
Between means of tests	9	273,409	30,379	5.899[a]
Between means of observers	4	18,009	4,502	0.874[b]
Remainder	36	185,401	5,150	

[a] Highly significant.
[b] Not significant; $P = 0.05$.

The second factor, mentioned above, which deals with the precision of an assay is analyzed in Tables IV and V. The figures in Table IV are from the confidence limits calculated from the results obtained by each observer in the first four columns, and in column "C" from the confidence limits calculated from the results obtained when the combined reading of the lines for the four observers was used. The confidence limits were calculated as per cent of the determined potency and figures given in the body of the table represent the difference between the upper and lower limits. For example, the result obtained by observer 1 in test 1 was 939 International Units per gram with confidence limits at $P = 0.05$ of 66 to 152% of this value and 152 minus 66 is 86.

Table V presents an analysis of variance of the figures in Table IV. The variance ratio for "means of observers" is significant and points to real differences among the totals of columns in Table IV. This indicates a real difference in the degree of precision among the four observers and those where observations were combined. However, if column 5 of Table IV is omitted there is still a significant difference in precision obtained by the four observers.

TABLE II.—COMPARISON OF ACCURACY OBTAINED BY FOUR OBSERVERS IN TEN ASSAYS OF THE INTERNATIONAL STANDARD

Tests	Observers 1	2	3	4	C	Totals
	Differences between Determined and True Values					
1	61	136	14	35	4	250
2	78	348	119	469	302	1316
3	169	32	185	114	119	619
4	28	94	22	17	3	164
5	287	180	220	69	204	960
6	126	43	38	124	45	376
7	20	146	49	27	25	267
8	86	125	45	133	91	480
9	156	233	193	216	255	1053
10	63	128	82	140	8	421
Totals	1074	1465	967	1344	1056	5906

TABLE IV.—PRECISION OBTAINED BY FOUR OBSERVERS AS MEASURED BY THE SIZE OF THE CONFIDENCE INTERVALS FOR $P = 0.05$

Tests	Observers 1	2	3	4	C	Totals
	Confidence Intervals					
1	86	85	92	79	79	421
2	147	151	210	151	146	805
3	92	118	102	74	75	461
4	79	76	92	69	72	388
5	85	66	85	71	69	376
6	73	92	110	69	69	413
7	69	93	75	76	67	380
8	73	69	77	78	60	357
9	107	96	130	82	89	504
10	204	159	140	110	130	743
Totals	1015	1005	1113	859	856	4848

The assay of the International Standard in terms of itself represents the best possible conditions for precision under which a biological test can be conducted since the solutions to be compared are identical in potency, their potency is known and experience has shown the proper doses to be administered to give the most favorable responses on the dosage response curve. Therefore the precision obtained should be at its maximum when other conditions are equal.

TABLE V.—ANALYSIS OF VARIANCE OF FIGURES IN TABLE IV

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square	F
Total	49	59,184		
Between means of tests	9	45,576	5064	22.6[a]
Between means of observers	4	4,902	1226	5.47[a]
Remainder	36	8,076	224	

[a] Highly significant.

The precision for samples of unknown potency is not so good. This is revealed by the figures in Table VI which compare the size of the confidence intervals for determinations of the International Standard with those for samples of "unknown" oils in the same assays. Twenty-four assays which included a comparison of the standard with itself and the determination of the potency of a sample are included. The figures in the table again give the size of the confidence intervals as per cent, as in Table IV. The average size of the confidence interval for the standard is 90.5 and for the samples is 106.5 but the difference between the two means is barely significant at $P = 0.05$ by the "t test."

DISCUSSION

A recent note in *The Analyst* and *Chemistry and Industry* (1) shows that British workers were able, on the average, to obtain confidence limits for vitamin D assay by the rat from 54–170% to 87–115%. It is not stated which results were applicable to tests in which litter mates were distributed among the groups nor how many animals were used. At least two dose levels of standard and samples were used, however.

According to Irwin (3), who quotes from reports of the British Pharmacopeia Commission, the limits of error (confidence limits) for vitamin D assays by the line test when $P = 0.05$ will be when 40 rats are used about 68–146%, and 78–129% when 80 rats are used. It is assumed that two doses are to be used for each of the standard and sample.

Our results under the best conditions, i.e., comparisons of the standard, show limits between 51% and 196% and 75–133% with the average 65–155%.

TABLE VI.—PRECISION OBTAINED IN ASSAYS OF THE INTERNATIONAL STANDARD AND SAMPLES OF COMMERCIAL FISH OILS AS MEASURED BY THE SIZE OF CONFIDENCE INTERVALS FOR THE ASSAYS[a]

Test	Standard	Sample	Test	Standard	Sample
51	58	71	63	118	111
52	99	99	64	99	85
53	95	115	65	70	133
54	92	126	73	61	88
55	118	126	74	70	75
56	103	116	75	67	66
57	95	52	76	73	181
57-b	73	91	77	70	70
59	99	117	78	79	62
60	118	135	79	145	148
61	79	151	80	76	117
62	131	131	82	85	92

[a] Mean confidence interval for standard = 90.5, for samples = 106.5.

Results that are to be used in regulatory work as the basis of prosecution or other enforcement action, however, should be known with greater precision. The practice is to repeat the assay on a sample on which enforcement action may be taken and to combine the results of the separate determinations of potency. The weighted mean potency with its limits of error are then used in judging the potency of the sample.

It is, of course, desirable to obtain this degree of precision with a minimum of work and expense.

It is interesting to note that no detectable increase of accuracy was obtained by having four observers independently record the degree of healing. This is shown by the figures in Table II and the analysis of variance in Table III where there is no significant difference in the results obtained from the readings of the four observers treated independently and as combined observations in the assay of International Standard against itself. There is, however, a significant difference in the precision of individual observers as measured by the size of the confidence intervals, Tables IV and V, but the results of the combined efforts shown in column C, Table IV, are not shown to be better than those obtained by the individuals when calculated separately. For example, the results of Observer 4 were as precise as those from the combined results of all four observers. It is known that Observer 4 had somewhat longer experience in assessing the degrees of healing of the bones than the other three.

It is, of course, less expensive to have the one person who conducts the experiments do the whole of it and use his own readings for calculation. It may be concluded, therefore, that there is no economic justification shown in this work for a requirement that more than one observer examine the bone sections for healing.

It should be noted that precision can be increased by distributing litter mate rats equally among the various doses and tests.

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Nitrites XIII. Sodium Theophylline Alkylglycollate Nitrate*

By HARRY K. IWAMOTO, JOSEPH G. BIRD, C. JELLEFF CARR, JOHN B. HARMON and JOHN C. KRANTZ, Jr.

A new solubilizing agent for theophylline is reported. Combinations of theophylline with isobutylglycollate nitrate are water soluble and produce slightly alkaline solutions. The depressor response of theophylline upon intravenous injection in the anesthetized dog is prolonged owing to the glycollate nitrate.

IN PREVIOUS work (1) we reported the combination of theophylline with sodium glycinate and its advantages in the administration of theophylline. It is well known that alkali salts of organic acids and amines render theophylline water soluble.

During the course of investigation of a series of nitric acid esters of alkylglycollates it was observed that these esters in aqueous solution gave rise to uniformly high acidity (*p*H 2.0 to 3.0). The sodium salts of these esters were prepared by the addition of one mole of sodium hydroxide. These solutions were capable of dissolving an equimolar quantity of theophylline. By the addition of a slight excess of alkali to restore the *p*H, to 8.5 to 9.0, the aqueous solutions were considerably more stable. It is well established that the esters of malonic, acetoacetic, and cyanoacetic, through enolization, are sufficiently acidic to form salts when treated with sodium hydroxide. These salts are soluble in water. It appears quite likely that a similar condition may exist, and it is our opinion that the structure of sodium theophylline alkylglycollate nitrate is best represented by the equilibrium: (see page 222).

* Received July 16, 1949, from the Department of Pharmacology, School of Medicine, University of Maryland, Baltimore.

$$O_2NOCH_2COOR \xrightarrow{NaOH} NaO_2NOCHCOOR \xrightarrow{C_7H_8N_4O_2\cdot H_2O\ (Theophylline)} [Na^+ + {}^-O_2NOCHCOOR \rightleftarrows C_7H_7N_4O_2^- \rightleftarrows H^+]$$

This combination affords the solubilizing of theophylline with a compound which elicits synergistic vasodilatation. The purpose of this paper is to describe certain of the pharmacodynamic studies made with this theophylline combination.

EXPERIMENTAL

Preparation.—Isobutylglycollate nitrate, 35.4 Gm. (0.2 mole) and theophylline monohydrate, 39.6 Gm. (0.2 mole) were separately dissolved in sufficient amount of 95% alcohol with heat if necessary to effect complete solution of theophylline. A carbonate-free solution of 6 *N* NaOH, 36 cc. (0.2 mole), was added to the alcoholic ester. Theophylline solution was then added to the sodium salt solution of the alkylglycollate nitrate. On chilling in an ice bath, crystals of the combination product were obtained. The crystals were collected on a Büchner funnel, washed with a chilled mixture of alcohol and ether in equal parts of each, and dried in a vacuum desiccator. On the removal of the solvent under reduced pressure, the remainder of the same material was obtained from the filtrate. The yield, except for the manipulative losses, was nearly quantitative.

In a similar manner, the following additional alkyl derivatives were made: *n*-butyl, *n*-amyl, *n*-hexyl, and *n*-heptyl.

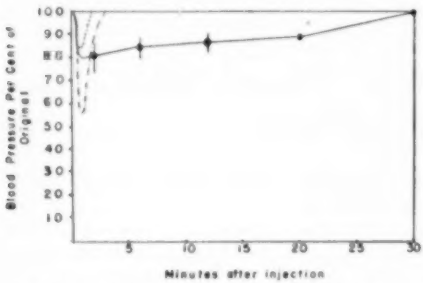

Fig. 1.—Comparison of depressor activity of theophylline derivatives upon intravenous injections into dogs. Solutions contained 1% theophylline and 1% of the solubilizing agent. Dose in all cases was 0.5 cc. per Kg.
●——●, theophylline with sodium isobutyl glycollate nitrate; standard deviations in 5 animals shown. — · — · — · —, theophylline with ethylene diamine (Aminophylline). - - - - - -, theophylline with sodium glycinate (Theoglycinate).

Properties.—The material when prepared in the above manner was a straw-colored or nearly white powder. The powder has a slightly bitter taste.

The dry powder dissolves in water without any difficulty up to 5 to 6% (*w/v*) at ordinary temperature, irrespective of the $-CH_2$ difference between each homolog in the members studied. Interpreted on an equimolar basis, it follows the general trend of diminishing water solubility with increasing molecular weight.

The water solutions containing 2 to 5% (*w/v*) of these theophylline combination products have a *p*H range of 8.5 to 9.0 and are stable. Neither decomposition nor loss in vasodepressor potency was observed in the solutions after standing for one month.

Blood Pressure Studies (Dogs).—The depressor responses to sodium salts of the various alkylglycollate nitrates without theophylline was studied in etherized dogs. The ethyl, *n*-propyl, isopropyl, *n*-butyl, and isobutyl esters were injected intravenously into 13 animals (6.5 to 7.6 mg./Kg. on an equimolar basis). The blood pressure of the 13 animals was immediately reduced to a mean value of 75.6% of the pre-injection levels with a standard deviation of 6.5%.

The isobutyl compound in combination with theophylline in solution containing 1% of each was injected into 5 dogs. A volume of 0.5 cc. per Kg. was employed. Similar doses of theophylline in combination with ethylenediamine and with sodium glycinate were injected into other dogs for comparison of duration of action.

The data in Table I and in Fig. 1 show the prolonged depressor effects in dogs of theophylline in combination with sodium isobutylglycollate nitrate. Figure 1 also shows responses obtained by the injection of the other theophylline combinations.

TABLE I.—DEPRESSOR ACTIVITY OF THEOPHYLLINE WITH SODIUM ISOBUTYLGLYCOLLATE NITRATE

Dog No.	Depression of Blood Pressure, % of Initial Time After Injection, Min. 2	6	12	20	30
1	81.6	83.0	87.5	90.5	90.5
2	71.6	76.9	84.2	..	112
3	88.0	88.8	89.5	97.8	97.0
4	83.2	86.2	92.4	...	...
5	75.3	85.2	82.1	78.6	...
Mean	80.0	84.0	87.1	89.0	100
Standard deviation	6.5	4.9	4.1	..	...

SUMMARY

A new theophylline combination has been prepared in which the sodium salt of isobutylglycollate nitrate has been employed as the solubilizing agent. The combination is water soluble and slightly alkaline in aqueous solution (*p*H 8.5 to 9.0). The depressor response of theophylline upon intravenous injection in the anesthetized dog is prolonged owing to the action of the glycollate nitrate.

REFERENCE

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Photosensitivity of Sunscreens*

By ELEANOR CHRISTENSEN† and ARTHUR C. GIESE†

The changes in absorption of ultraviolet light were determined for fifteen sunscreens following two-hour exposures to a sunlamp. Some showed increases (ethyl-*p*-aminobenzoate, amyl and phenyl salicylate, menthyl anthranilate, 2-naphthol-6-sulfonic acid, tannic acid, and phenol), some showed decreases (benzyl-salicylate, homomenthyl cinnamate, benzylacetophenone), some showed little change (ethyl-*p*-dimethyl-aminobenzoate, menthyl, homomenthyl, and isoamyl salicylates). The data indicate that under the test conditions employed many sunscreens show relatively high stability withstanding many minimal erythemal doses without much change.

SUNBURN PREVENTIVE preparations contain among other materials constituent chemicals called sunscreens with a marked absorption in the sunburn-producing region of the sunlight (1). Of the various properties desirable in the sunscreen one is a high resistance to photodecomposition on exposure to sunlight. If the sunscreen loses its power to absorb the sunburn-producing radiations of sunlight, it is rendered useless. The sensitivity to light has often been mentioned in the literature (2), but no systematic investigation seems to have been made. It was the purpose of the present study to determine the photosensitivity of representative sunscreens and to measure the changes in their absorption following irradiation.

EXPERIMENTAL

The compounds selected for this work were the ones reported to absorb most effectively that part of the sun's spectrum which has maximal erythemal efficiency (4). All solutions were made up to 0.001% in absolute alcohol except tannic acid which was dissolved in distilled water. In each case the solution, contained in a small cell covered with a quartz lid sealed on with silicone grease, was stirred continuously during exposure with a magnetic flea. The source of radiations was a G. E. 275-watt R S Sunlamp, a type which plugs directly into the 110 a.-c. line. Such a lamp, in addition to heat and some visible light, gives off ultraviolet rays approximating those of the sun. While a lamp emitting shorter wave lengths would be more effective photochemically, interest in the present instance centers mainly in resistance of the sunscreens to sunlight.

The intensity of the lamp was about 175–200 ergs/mm.²/sec. for different parts of the field as determined approximately with a Hanovia U.V. meter. Such a lamp at 24 in. is supposed to give a sensitive skin a minimal erythemal dose (MED) in about four minutes. As it was used at a distance of 9 in. from the sample in the cell, it is likely that in the course of an hour a dosage of several MED would have been given to the sample. In two hours the sample would probably have been exposed to the maximum dosage which an individual even under favorable conditions is likely to obtain in nature (4). Measurement of the absorption of ultraviolet light by a solution in a 0.5-cm. quartz cuvette was determined using a Beckman spectrophotometer. After irradiation for an hour another measurement was made which was repeated after a second hour exposure.

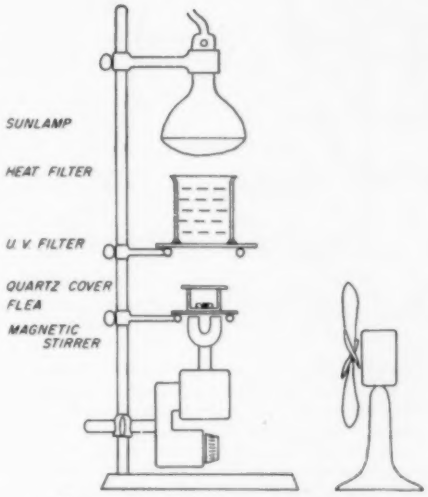

Fig. 1.—Assembly of equipment used.

The cell containing the solution was protected from the heat of the lamp by interposing a 600-ml. beaker of water to serve as an infrared filter. The bottom of the beaker was cut off and in its place, with deKhotinsky cement, was attached a 2-mm. Corning No. 9700 filter which transmits radiations of 280 mμ and longer. The entire assembly (Fig. 1) was cooled by the use of a fan. The solutions in the irradiation cell never rose above 27° after two-hour exposures; therefore thermal effects may be neglected.

The data gathered are briefly summarized in Table I and in Figs. 2 to 10 for those compounds which showed the greatest changes or the changes of greatest interest. Examination of the data

* Received August 18, 1949, from the Department of Biological Sciences, Stanford University, Calif.

† We are indebted to Professor T. C. Daniels of the College of Pharmacy, the University of California, for the sample of ethyl-*p*-dimethyl-aminobenzoate and to the late Julian M. Wells of the same school for many of the other compounds.

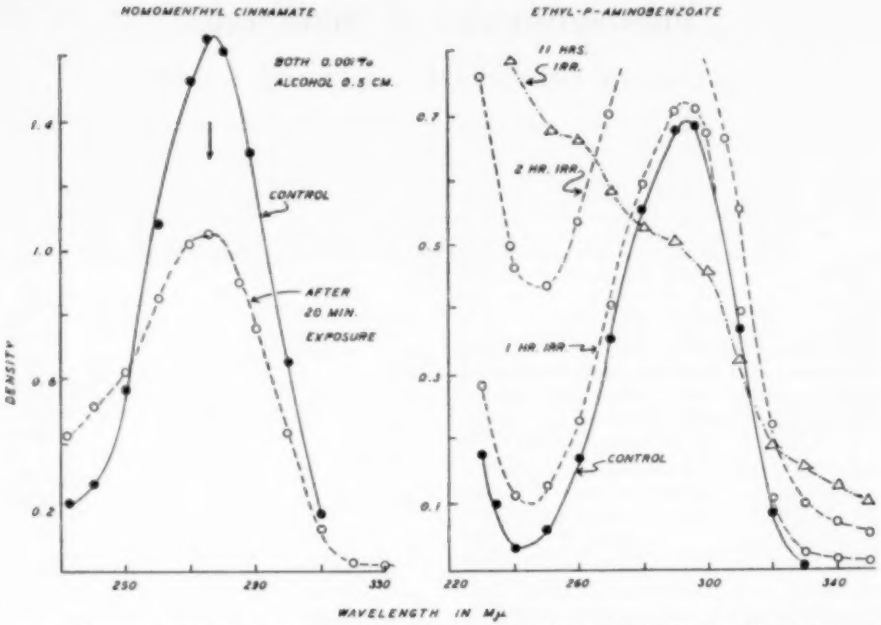

Fig. 2.—Change in absorption of homomenthyl cinnamate and ethyl-*p*-aminobenzoate. The values went off the scale in two hours in the latter case.

shows that the effect of irradiation of the sunscreens is quite variable, some substances showing increased absorption, some decreased, some no change at all, for the exposure time used.

Among the sunscreens which were previously judged to be very good on the basis of their high absorption in the region of maximal erythemal effectiveness of sunlight (1, 3), *p*-aminobenzoate (Fig. 2) shows increased absorption following continued irradiation, while homomenthyl salicylate (Fig. 3) shows only a slight fall in absorption at the short end of the spectrum including the peaks, even after a two-hour exposure. These sunscreens therefore pass the stability test.

When sunscreens show a decreased absorption after irradiation it is often a differential change, i.e., the peaks may decline but the troughs in the absorption spectrum may show a rise. This is illustrated in Fig. 2 for homomenthyl cinnamate, Fig. 5 for benzyl salicylate, and Fig. 6 for benzylacetophenone as well as to a lesser extent in others. When sunscreens show an increase in absorption after exposure it tends to be general all along the spectrum as seen in Fig. 2 for ethyl *p*-aminobenzo-

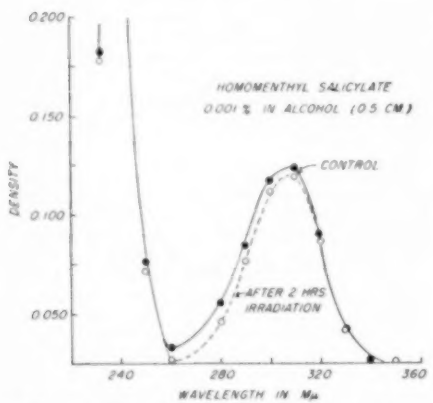

Fig. 3.—Slight decrease in absorption by homomenthyl salicylate after two hours exposure to the sunlamp.

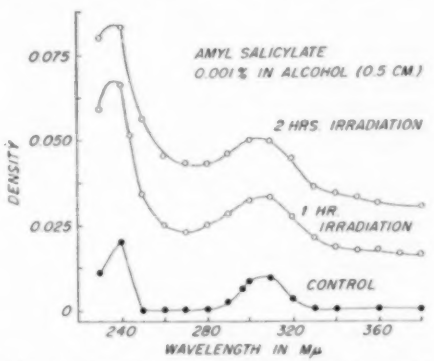

Fig. 4.—Increases in absorption shown by amyl salicylate after exposure to the sunlamp.

ate, Fig. 4 for amyl salicylate, Fig. 8 for tannic acid, and Fig. 9 for phenol. Phenol has been included in these studies because of its presence in carbolated petrolatum (3).

The experiments with menthyl anthranilate are interesting in so far as this compound was judged useless in our first study because it does not have a very high absorption in the region of 2970 Å. and was included here only as an example of this family of compounds. On irradiation it shows a distinct rise in absorption which is especially marked in the 2970 Å. region: for example, the initial density was 0.014; after one-hour exposure it was 0.045; after two hours 0.085. Thus a feeble sunscreen may be converted into a fair one on exposure. How-

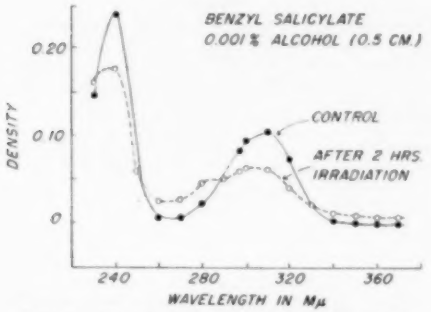

Fig. 5.—Changes in absorption by benzyl salicylate. Note fall in peaks and rise in valleys leading toward a relatively nonspecific absorption.

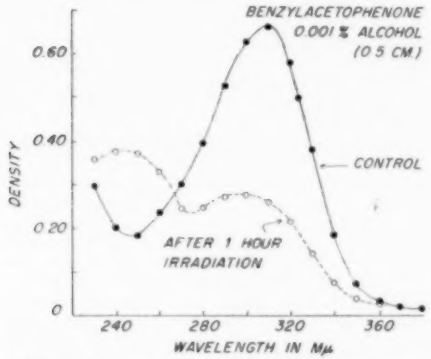

Fig. 6.—Change in absorption by benzylacetophenone after one-hour exposure to the sunlamp. Note gain in the short ultraviolet and loss in the erythemic region.

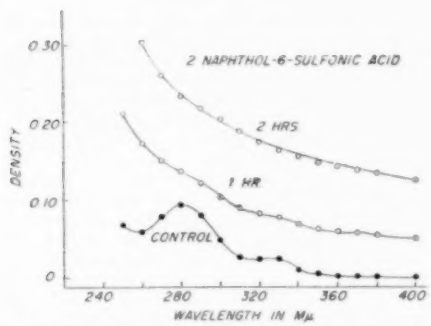

Fig. 7.—Great increase in absorption by 2-naphthol-6-sulfonic acid after exposure to the sunlamp for two hours. Note loss of specific absorption.

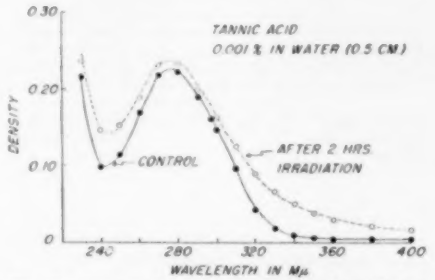

Fig. 8.—Increased absorption by tannic acid after irradiation with sunlamp.

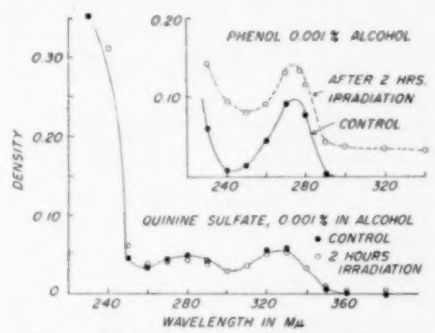

Fig. 9.—Slight change in absorption by quinine sulfate and marked increase in absorption by phenol after two hours' exposure to the sunlamp.

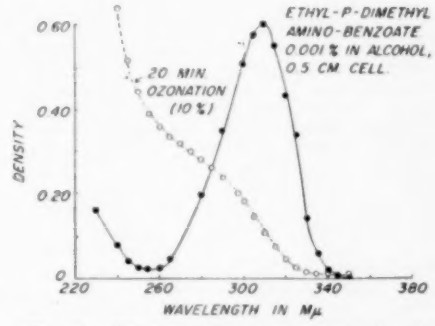

Fig. 10.—Effect of ozonation on absorption by ethyl-*p*-dimethyl-aminobenzoate. Note loss of specific peak at 310 mμ and rise in absorption in the short ultraviolet wave lengths.

TABLE I.—EFFECT OF SUNLAMP ON SUNSCREENS

Group	Examples	Effect of 2-Hr. Exposure
p-Aminobenzoates	Ethyl-*p*-dimethyl-amino-benzoate	Little change, slight fall at maximum
	Ethyl-*p*-aminobenzoate	Great increase
Salicylates	Menthyl-	Little change, slight increase below 2400 Å.
	Homomenthyl-	Slight fall
	Isamyl-	Little change
	Amyl-	Great increase
	Phenyl-	Slight increase
	Benzyl-	Fall in peaks, rise in valleys
Anthranilates	Menthyl anthranilate	Increase over entire scale
Pyrrones	Homomenthyl cinnamate	Fall
Acetophenones	Benzylacetophenone	Great decrease
Naphthol sulfonic acids	2-Naphthol-6-sulfonic acid	Great general increase but a loss of specific absorption
Tannic acid	Tannic acid	Rise
Quinine sulfate	Quinine sulfate	Slight fall
Phenol	Phenol	Rise

ever, it is doubtful whether such a sunscreen would be advisable for use in a preparation because of its low initial protection.

The differences between the photosensitivity of the various sunscreens may be considered as one of degree—some showing high sensitivity, some low. In the latter case the absorption may actually rise at first in the erythemic region but ultimately it should fall as the chromophore is altered. This is illustrated after an eleven-hour irradiation of ethyl *p*-aminobenzoic acid as shown in Fig. 2. It will be remembered that this compound showed a rise in absorption after a two-hour irradiation (Fig. 2).

It seemed of interest to determine whether the erythemic radiations had some specific effect on the sunscreens or whether shorter ultraviolet radiations would have the same effect. A few of the sunscreens were accordingly exposed to the radiations from a Sterilamp. About 85% of the radiations of this lamp are at λ 2537 Å. and the intensity was found to be about 90 ergs/mm.²/sec. Phenyl salicylate and homomenthyl salicylate tannic acid and carbolated petrolatum showed great increases in absorption in twenty minutes, whereas quinine sulfate and homomenthyl cinnamate showed a considerable decline in the same period of time. It would therefore appear that the changes are in the same direction as those produced by the sunlamp and, if anything, are produced even more readily in the latter case as one might expect from the larger energy content of the quanta at the dominant wave length.

As to the chemical nature of the changes which lead to changes in absorption, little can be said without further experiments. However, on the basis of similar changes in amino acids and their analogs the conclusion was drawn that oxidations occur. The first oxidations lead to increases in absorption, but continued oxidations result in compounds which show a decline in specific absorption in the ultraviolet (5). It is likely that similar changes occur here. One experiment was performed using ozone as an oxidizer for a solution of ethyl-*p*-dimethyl-aminobenzoic acid, and, as shown in Fig. 10, the specific absorption is completely lost, indicating that the chromophoric group has been altered or destroyed.

In conclusion it is apparent that some sunscreens are quite resistant to sunlight ultraviolet and resist many MED of sunlight before they show any change; some show increased absorption, some decreased absorption. In use the sunscreen may be present in a sunburn preventive preparation along with many other chemicals which may alter its photosensitivity, e.g., the antioxidants sometimes used might retard the changes produced by absorption of light. Only experiments would furnish the necessary information but it would be necessary to perform the experiments for each particular combination of reagents used in each preparation.

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Bioassay of Purified Glycosides Using the Inotropic Effect on Isolated Hypodynamic Cat Myocardium*

By JOHANNES IPSEN, Jr., and WALLACE F. WHITE†

Instead of using death as the end point as in the official cat assay of digitalis, it is possible to estimate potency of glycosides by determining relative threshold inotropic doses on isolated heart muscle which has been subjected to an unfavorable environment. This method approaches more closely the intended use of cardiac glycosides in therapy since improvement of hypodynamic heart tissue is accomplished in both cases. The error of this method, however, appears to be of the same order as the official cat method. Estimation of potency by a second method using the slope of the concentration response curve with standard alternated with unknown is more cumbersome and no more precise than the threshold method. Of eleven purified cardiac glycosides studied, ten appeared to act qualitatively the same, differing only quantitatively.

In previous communications from this department (4–6), the experimental details were reported on the adaptation of the isolated papillary muscle preparation of Cattell and Gold (2) for quantitative studies. It was also reported that the various inotropic glycosides could be mutually substituted in isodynamic equivalent quantities. The equivalence factor was related to ouabain, and the relative potencies obtained by substituting in the hypodynamic heart preparation were compared to the relative potencies found by Chen (3). He used the mean lethal dose in intact cats as a basis for comparison.

The purpose of the present paper is to revise the biostatistical basis of determining the relative potencies of drugs acting on the hypodynamic mammalian myocardium. It was felt that the method of estimating the potency used previously (5) contained a certain bias which obscured the estimate of the precision of the potency determination.

EXPERIMENTAL

The various stages of the hypodynamic condition of the myocardium are best illustrated on a diagram relating the amplitude of the muscle to time (Fig. 1). Four stages can be distinguished. First—the decrease of amplitude during the period of work in *deficient* oxygenated Ringer's solution as explained in an earlier paper (6). Second—the equilibrium or turn over, when the Ringer's solution is replaced by a solution of the inotropic drug. Third—the increase in action when the concentration of glycoside is increased logarithmically at the same rate. Fourth—the final stage when the concentration of the drug has reached the toxicity level: the increase in amplitude stops and there is a rapid decrease in action due to toxic rigor.

* Received August 5, 1949, from the Department of Pharmacology, Yale University School of Medicine, New Haven, Conn.

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Theoretically, doses of the compounds can be compared at three stages:

Stage 2. The minimum dose which shows a positive inotropic effect.

Stage 3. The dose of one drug which can substitute for a dose of another drug isodynamically at this stage, meaning that the work increases at the same rate as before the substitution.

Stage 4. The dose which is required to cause a toxic effect on the isolated myocardium.

We are primarily interested in observing the therapeutic effect, and the toxic effect is not measured in this connection, since it would require a different experimental approach; hence, for practical purposes, potency determinations must be based on measurements taken in Stages 2 or 3.

Determination of the Threshold Dose.—The determination of the minimum dose which causes a positive inotropic effect may be an isolated assay, but it is preferable to combine it with Stage 3. The minimum effect can only be determined for one particular drug in the same muscle preparation while two drugs can be compared on the ascending curve in Stage 3.

The procedure is to allow the isolated papillary muscle to work in deficient Ringer's solution until the amplitude is below one-half of the initial value. This may take one to several hours. The period of time required seems to depend mostly upon the quality of the preparation. Some muscles will reach as far as one-tenth of the initial value within a little more than one hour while other preparations will not go far below 60% of the maximum in the course of several hours. It is advisable to start the assay at least after three hours, whatever the level of work is at that moment. If a low amplitude is obtained in shorter time the application of the drug can start then. If the amplitude decreases too much the preparation may be so exhausted that the drugs are ineffective.

The determination of the minimum dose is merely a titration where increasing doses are used until the desired effect is obtained. As the starting dose, a solution containing the equivalent of 1.0 μ of ouabain per 100 cc. may be used. The solution used as a vehicle for the drugs is the same deficient Ringer's solution used in obtaining the hypody-

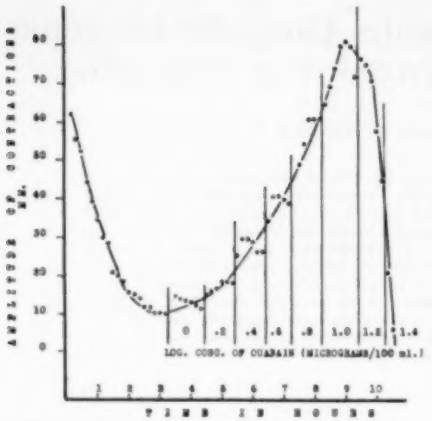

Fig. 1.—The four stages of the hypodynamic myocardium. Stage 1: 0–3 hours; Stage 2: 3–4½ hours; Stage 3: 4–9 hours; Stage 4: over 9 hours.

namic muscle. This glycoside solution replaces the Ringer's solution, and the amplitude is recorded in the camera every fifteen minutes. The steady diminution in amplitude observed up to then will either slacken or end, so that the amplitudes are the same throughout one hour. Hourly thereafter, the bathing solution is replaced by one having a greater concentration of the compound under test. The best increase in concentration will be 25% per hour (or a log interval of 0.10), e.g., the concentration will be 1.00, 1.25, 1.60, 2.00, etc. The necessary presumption is that some pilot experiments or other information have yielded a general idea of the approximate potency of the drug in relation to ouabain.

The end point of the titration is indicated by an increase of the size of the amplitude which may be barely distinguished from the amplitude in the hour interval before. If the next dose causes a continuation or further increase of the rising amplitude, the end point is assured.

The minimum effective dose is then defined as the geometric mean of the highest concentration showing no increase of amplitude and the lowest dose causing an increase in amplitude. It follows from this definition that an experiment is successful only if doses of the same drug on both sides of the end point are observed. If the amplitude starts to increase immediately after application of the first dose the limiting value is not observed. The fact that several doses should be applied to each muscle preparation with at least one hour interval, limits the range of concentrations which can be tested. In other words the approximate minimum effective dose must be known beforehand.

Most of the experiments recorded in our protocols were not performed with special regard to the threshold dose determination, but those which did fulfill the conditions of a successful experiment as defined above are presented in Table I.

These experiments should not be considered optimal for the purpose of determining the relative equivalence of the various glycosides. They were performed mainly to study the nature of the inotropic effect, but the data which are extracted from them and shown in Table I are sufficiently adequate for an analysis to indicate an appropriate design for a bioassay of two glycosides with the therapeutic threshold dose as a basis of comparison.

A rough way to compare a test drug with ouabain would be simply to subtract the mean log dose of ouabain from the mean log dose of the unknown, but this procedure would not take into regard the fact that the threshold dose is correlated with the degree of depletion of the muscle at the moment when the drug is supplied. The nine experiments with ouabain demonstrate that the lower the amplitude the lower the threshold dose. Since the absolute values of the amplitude at Stage 2 (*L* in Table I) are dependent upon the degree of magni-

TABLE I.—DATA FOR DETERMINATION OF EQUIVALENCE FACTOR USING THRESHOLD DOSE OF GLYCOSIDES

Drug	Expt. No.	μg./100 Cc. *A*	*B*	Millimeters *H*	*L*	*X*	*Y*	*E.* Factor
Ouabain	10	0.8	1.0	81	7	−0.05	−1.02	
	12	0.8	1.0	41	7	−0.05	−0.69	
	11	0.8	1.0	93	16	−0.05	−0.68	
	9	1.0	1.6	52	12	0.10	−0.48	
	8	1.0	2.1	28	5	0.16	−0.66	
	16	2.5	3.6	92	16	0.48	−0.68	(0.00)
	40G	3.2	3.2	10	5	0.50	0.00	
	41G	3.2	4.0	33	9	0.55	−0.43	
	17	5.0	5.3	33	15	0.71	−0.08	
Digitoxin	13E	2.8	4.0	63	22	0.52	−0.27	
	3G	6.3	8.0	88.5	30	0.85	−0.29	0.346
	1E	6.3	10.0	78.5	24	0.90	−0.36	
Convallatoxin	18G	1.9	2.4	41	11	0.33	−0.44	0.073
	20G	3.0	3.8	25	8	0.53	−0.33	
Cerberoside	26G	15.6	19.5	42	21	1.24	0.00	0.617
Cerberin	16G	1.4	1.9	62	42	0.22	0.32	−0.624
Oleandrin	11F	2.5	4.0	84	17	0.50	−0.53	0.243
Scillaren A	6F	2.4	2.4	60	14.5	0.38	−0.50	0.102
Uzarin	28G	146.0	183.0	18	4	2.21	−0.54	1.960

A = highest ineffective dose; *B* = lowest effective dose; *H* = highest contraction; *L* = lowest contraction; $X = \frac{1}{2}(\log A + \log B)$; $Y = \log L/(H - L)$.
E. Factor = the log dose of the drug which will substitute for 1.0 μg. of ouabain (11% H_2O).

fication of the optical lever, we correct for mechanical conditions of observation by a transformation expression in which the maximum amplitude observed for the particular muscle preparation enters. Several trials with various functions have suggested that the function which is designated as Y in Table I is rectilinearly related to the threshold dose with the same variation at any level within the range investigated.

If Y is defined as the log of the ratio of the lowest amplitude to the difference between the highest and lowest value, and X defined as the logarithmic mean of the highest ineffective and the lowest effective dose, a least square fit of the available data gives the following relation between threshold dose and level of work of the myocardium:

$$X = M_I + 0.623 + 0.69\ Y \qquad (1)$$

where M_I is the log equivalence factor for a given glycoside to ouabain. Antilog M_I is the number of micrograms of a glycoside which can substitute for 1 μg. of ouabain at a given level of work. In a previous communication (6) a similar equation was used to relate the concentration of drug and the response, but we now consider this application too general. We confine the relation to the determination of the concentration of drug which is necessary to provide a threshold effect at a certain level of depletion of the heart muscle. The equation does not determine the size of the effect, because the effect will be counteracted in part by the rate of deterioration of the individual myocardium which is an unknown factor. An attempt to correlate the degree of increase to the lowest effective dose did not yield further information.

Equation 1 was used to determine the equivalence factor for a given drug. The mean of X, $\bar{X}$ and the mean of Y, $\bar{Y}$ are computed for each set of experiments with the glycoside, and used in Equation 2 which follows:

$$M_I = \bar{X}_s - \bar{X}_o - 0.69\ (\bar{Y}_s - \bar{Y}_o)$$

where

$$\bar{X}_s = 0.261 \text{ and } \bar{Y}_s = -0.524$$

$$M_I = \bar{X}_s - 0.623 - 0.69\ \bar{Y}_s \qquad (2)$$

The constants of the equation are determined from the nine experiments with ouabain in Table I. The values may vary slightly for another set of data which should be procured in each laboratory that wishes to utilize the method for the purpose of practical assay.

The error involved in the method is determined by the variation of the X's for a given value of Y. An estimate of this variation is given by the analysis of variance in Table II.

The analysis of variance shows that the error is reduced to about one-half by correcting the threshold dose for the degree of depletion of the muscle as indicated by Y. The inherent error will be reduced if the coefficient of Y is determined from a large number of data. The present data are too few to ascertain a value for the error, but it may be anticipated that its order of magnitude will be the square root of 0.0433 or 0.208.

It is not possible to apply both standard and the unknown to the same myocardium preparation by this method, but it is entirely possible, according to the experience in this laboratory, to prepare two papillary muscles on the same day from the same cat's heart. It is highly probable that the error of a determination of relative potency would be reduced markedly by using such paired experiments; one muscle for determining the threshold value of the standard and the other for determining the threshold value for the unknown drug.

TABLE II.—ANALYSIS OF VARIANCE OF EQUATION 1

	D. F.	Sum of Squares	Mean Square
Variance of X (threshold values)	11	0.8217	0.0747
Mean regression coefficient	1	0.3963	0.3963
Variance of regression coefficient	2	0.0790	0.0395
Error (X reduced by Y)	8	0.3464	0.0433

Determination of Equivalence by Substitution on the Stage of Increasing Contraction.—The stage of increasing contraction is characteristically produced by increasing the dose from the threshold level at a constant percentage rate per hour. By this scheme the natural deterioration of the muscle tissue is exceeded, and the curve of muscle contraction plotted against time will form a rectilinear curve except for the lower and upper portions where the curve smooths off horizontally.

Figure 2 illustrates schematically the procedure of comparing the two drugs in Stage 3. At time 0 when the inotropic effect is apparent, the relevant dose of the standard (ouabain) is applied. After one hour the solution is replaced by a solution of the unknown. The concentration of this solution is assessed, first from the *assumed* equivalence factor, and second by the time elapsed since 0 hour.

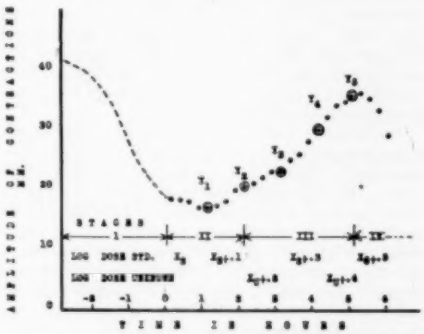

Fig. 2.—Comparison of glycosides at stage of increasing contraction, Stage 3. Semischematic diagram illustrating the fact that increase in dose is determined by the time. Hence, the slopes $Y_4 - Y_3$ and $Y_2 - Y_1$ are both referred to the initial dose X_s.

If we assume an equivalence factor of 2.0 then log dose of unknown, x_u, equals log dose of standard, x_s, plus log of 2.0, or 0.3 (M). Further, since one hour has elapsed and the hourly log dose increase, i, is 0.10, we have log dose of unknown equals x_s plus M plus i, which equals $x_s + 0.4$ or $x_u + 0.10$.

Two hours after 0 hour the standard replaces the unknown. The concentration of the standard solution is now increased by $2i$ over the initial so that the dose is $x_s + 0.20$. Finally the second substitution with the unknown takes place three hours after 0 hour. The dose is now $x_u + 0.30$ or $x_s + M + 0.30$.

If the equivalence factor has been assumed correctly the increase per hour will be the same, in practice, meaning that the average increase for the unknown will have the same value as the average increase for the standard. If the unknown causes a smaller increase than the standard, the assumed equivalence factor is too small, and vice versa.

It is important to emphasize that this procedure only estimates the correctness of an assumed equivalence factor. Although two different doses of each drug are applied, the assay is not a classical "two-by-two" assay, because the size of the second dose of each of the standard and unknown is entirely determined by the elapsed time, so that we are in reality comparing *replicates of one dose of each drug*.

The correct equivalence factor can be determined biometrically by performing separate assays with the standard and the unknown and assume different factors so that some experiments show a higher slope for the unknown and some a higher slope for the standard. By relating the positive and negative differences of the slopes to the assumed equivalence factors, the factor which would give zero difference can be interpolated.

The steps involved in the treatment of the data are illustrated in the following numerical example. The first step is to calculate the average slope for each of the glycosides and compute the differences between them. In order to make the values obtained from separate experiments comparable, the differences in slope are divided by the mean of all four slopes. This mean is always computed from the numerical values in order to account for the cases where some of the slopes are negative, in which cases the mean might approach zero if not computed from the numerical values.

The slope at each dose is simply computed as the difference between the amplitude of contraction measured at the end of the period and the amplitude at the beginning of the period in which the given concentration of drug is applied. In some experiments because of changes in amplitude brought about by physical disturbances of the muscle, it was found advisable to average the last reading during a test period with the first reading at the next higher concentration. The slopes were then computed from these values.

The assumed equivalence factor in the example in Table III is equal to -0.15, since it is the difference $x_u - x_s$. The difference of the mean slopes for the unknown and the standard is positive, meaning that the assumed equivalence factor is too high, and that the ouabain doses should have been somewhat higher in order to produce a uniformly increasing curve with the given doses of convallatoxin. How much the equivalence factor should have been altered to accomplish a perfect substitution with zero difference of slopes cannot be calculated from this experiment separately. The d values for each experiment were corrected by division by the total mean slope for all experiments for each unknown drug.

Thus, the next step is to relate the M and d values from a set of experiments with the same drug substituted for ouabain alternately in Stage 3. Table IV contains the data from three experiments with oleandrin and ouabain.

The symbols in Table IV are explained in connection with Table III, except for the weight of d, w. If all assays had been performed with two doses of the standard and two doses of the unknown no weighting of d would be necessary, but since several of the experiments were performed with less or more doses than four a weighting is indicated, computed from the number of doses of the standard, n_s, and the number of doses of the unknown, n_u, as follows:

$$w = \frac{n_s n_u}{n_s + n_u} \qquad (3)$$

The problem is now to determine by a linear regression the equivalence factor which has the slope difference zero. If this interpolated M is designated M_o, we have

$$M_o = \bar{M} - \bar{d}/c \qquad (4)$$

where $\bar{M}$ and $\bar{d}$ are the weighted means of the M's and d's, respectively, and c is the slope determined by the least square method.

The error of M_o will depend largely upon the accuracy with which c is determined. Therefore, the final calculation of the equivalence factor should wait until a goodly number of assays have been collected. The c's from each set of experiments have been compared and if they vary within the error of the experiments a mean c can be computed with proper regard to the weight of each individual c.

The parallelism of 9 regression coefficients, c, obtained from sets of experiments with 9 glycosides

TABLE III.—MUTUAL SUBSTITUTION OF STANDARD AND UNKNOWN AT THE STAGE OF INCREASING CONTRACTION

Expt. No.: 3F			Log Dose,	Response							
Drug	T	D	Ti	y_1	y_2	y_3	y_4	y_5	Slope	$\bar{b}_s$	$\bar{b}_u$
Convallatoxin	0	1.0	0.00 (x_u)	28.5	32.0	..	..	..	7	..	16.5
Ouabain	1½	2.0	0.15 (x_s)	..	..	33.5	..	..	3	6.5	..
Convallatoxin	3	2.0	0.00 (x_u)	..	..	..	46.5	..	26	..	..
Ouabain	4½	4.0	0.15 (x_s)	..	..	..	..	51.5	10	..	..
									11.5 (mean slope)	= $\bar{b}$	=

$i = 0.10$ per hour; T = time of treatment (hours); D = dose in micrograms per 100 cc. solution.

$b_u = y_2 - y_1$ and $y_4 - y_3$. $b_s = y_3 - y_2$ and $y_5 - y_4$. $d = \frac{b_u - b_s}{\bar{b}} = +0.87$.

$\log M = x_u - x_s = -0.15$.

TABLE IV.—ASSUMED LOG EQUIVALENCE FACTOR (M) AND CORRECTED SLOPE DIFFERENCE ($\bar{d}$) FROM THREE SUBSTITUTION ASSAYS WITH OLEANDRIN AND OUABAIN

Expt. No.	Slopes Std.	Slopes Unk.	Mean Slope Std., b_s	Mean Slope Unk. b_u	Numerical Mean Slope $\bar{b}$	$\frac{b_u - b_s}{\bar{b}}$	Weight of $\bar{d}$ Assumed w	Weight of $\bar{d}$ Assumed M
62G	12	3	36	17.25	26.625	−0.70	1.0	0.11
	60	31.5						
11F	−9	12	−14	18	16	+2.0	1.0	0.40
	−19	24						
13F	3	7	25	7	19	−0.95	0.7	0.00
	47							

compared with ouabain, is shown in the analysis of variance in Table V. The variation of the c's was compared to the variation of the d's around the regression line, and also to the variance of the individual slopes, b, around their mean values b_u and b_s.

TABLE V.—ANALYSIS OF VARIANCE OF REGRESSION BETWEEN ASSUMED EQUIVALENCE FACTOR AND SLOPE DIFFERENCE

Variance	Degrees of Freedom	Sum of Squares	Mean Square
Individual slopes (b)	123	64.5554	0.5248
Slope differences (d) around regression line	59	27.4624	0.4655
Between regression coefficients (c)	8	4.0628	0.5079
Mean regression coefficient (C)	1	4.7094	4.7094

The experiments analyzed in Table V comprise 79 assays with 11 different glycosides tested against ouabain (with 11% H_2O). Two of the glycosides were only tested in replicates for the same equivalence factor, so that no regression coefficient could be determined in these cases.

The variance of the 9 regression coefficients was in perfect agreement with the expected variance as estimated from the deviation of the d's around the regression line. Again this variance is of the same order of magnitude as the variance of the individual slopes (b) around their means. Consequently the assays, although performed on 79 different heart preparations over a period of several years, are mutually compatible, and the regression can be assumed to be rectilinear.

The average regression coefficient, C, had the value 3.909. This figure was utilized in computing the equivalence factor, M_o, inserting C instead of c in Equation 4 together with the observed values of $\bar{M}$ and $\bar{d}$. The results are compiled in Table VI.

The precision of the evaluation of M_o is expressed by its variance, $s_{M_o}^2$, listed in the next to last column of Table VI. This quantity was calculated from the following expression into which the error terms in Table V enter:

$$\text{Variance of } M_o = s_{M_o}^2 = \frac{0.4655}{C^2}\left(\frac{1}{S(w)} + \frac{(\bar{d})^2}{4.7094}\right)$$

In a well-designed experiment where the mean difference, $\bar{d}$, approaches zero the inherent error will be of the order of:

$$\sqrt{0.4655/(3.909)} = 0.175$$

which is only slightly less than the inherent error of the threshold method, but much larger than the inherent error of the official method in the U. S. P. on intact cats, for which the inherent error is about 0.06. The inherent error of a bioassay is defined by Bliss and Cattell (1).

Comparison of Results on Isolated Myocardium and on Intact Cats.—Equation 1 elucidates a well-known clinical concept that digitalis only exhibits

TABLE VI.—COMPUTATION OF THE LOG EQUIVALENCE FACTORS FOR ELEVEN GLYCOSIDES (RELATED TO OUABAIN WITH 11% H_2O)

Drug	No. of Expts.	w	$\bar{M}$	$\bar{d}$	M_o	Variance of M_o $s_{M_o}^2$	Standard Error, %
Calotropin	4	4.1	−0.0500	0.6144	−0.2071	0.009869	26
Cerberin	4	3.5	−0.1800	0.6700	−0.3514	0.011605	28
Cerberoside	10	9.5	0.6703	0.3920	0.5701	0.004200	16
Convallatoxin	9	8.5	−0.1804	0.1987	−0.2312	0.003838	15
Cymarin	6	4.2	0.0000	0.6419	−0.1642	0.009915	26
Digitoxin	9	7.3	0.3679	−0.2254	0.4256	0.004502	17
Digoxin	8	7.5	0.3123	−0.0242	0.3185	0.004063	16
Oleandrin	3	2.7	0.1889	0.2351	0.1288	0.011639	28
Scillaren A	7	4.6	−0.1027	0.3733	−0.1982	0.007524	22
Thevetin	7	6.6	0.8115	0.3520	0.7215	0.005416	19
Uzarin	12	10.1	1.6415	−1.0327	1.9056	0.009909	26

w = sum of weights. M_o is expressed in relative potency in Table VII, third column.

inotropic action on heart muscle which works on a level which is a fraction of its maximal capacity. This relation between dose and fractional capacity determines the threshold dose necessary at a certain level of work, but does not determine the degree of the effect upon a myocardium, since the degree of inotropic effect will be the resultant of the drug action and the varying rate of deterioration of the muscle.

A comparison of the potency of different cardiotropic glycosides will only be valid, if the same equivalence factor is found when a glycoside is substituted for ouabain at *various levels of work* of the myocardium. We have determined the equivalence factor at various threshold levels and also at the whole range of activity from threshold level to toxic level, and these factors are presented in Table VII under the headings of "Threshold Method" and "Slope Method." The good agreement between the values obtained by the two methods is an argument in favor of the mutual replaceability of the cardiac glycosides.

TABLE VII.—EQUIVALENCE FACTORS OBTAINED BY THREE METHODS (MICROGRAMS OF GLYCOSIDE ISODYNAMIC TO 1 μG. OF ANHYDROUS OUABAIN)

Glycoside	Isolated Myocardium Threshold Method	Isolated Myocardium Slope Method	Lethal Dose in Cat, [Chen (3)]
Callotropin	...	0.71	0.96
Cerberin	0.26	0.50	1.27
Cerberoside	4.6	4.10	7.01
Convallatoxin	1.3	0.65	0.68
Cymarin	..	0.77	0.95
Digitoxin	2.5	2.90	2.80
Digoxin	...	2.30	1.99
Oleandrin	1.95	1.49	1.70
Scillaren A	1.4	0.70	1.26
Thevetin	...	5.90	7.66
Uzarin	90	90	39.50

The official cat method, U. S. P. XIII, determines the lethal dose for the entire animal under the assumption that the intoxication of the heart is the main responsible factor for the death. A direct test of this assumption is feasible by demonstrating that the toxic doses of various glycosides on the isolated cat myocardium have the same relative proportions as the lethal doses found in the intact cat.

A comparison of the equivalence factors obtained on the isolated myocardium and the equivalence factors found by the official method would illustrate whether the various glycosides have the same margin of safety. For this purpose the data presented by Chen (3) for the same drugs are given in Table VII. Chen's values are based on anhydrous ouabain so ours were corrected for the 11% of water content.

The agreement between the "Slope Method" results and Chen's data has been tested with the chi-square test, using the variances given in Table VI for the former method and the errors given in Chen's table to estimate the error of the difference. Only in the case of Cerberin was this difference significant, indicating that Cerberin may have a wider therapeutic range than ouabain. This needs verification by a direct test of the toxic dose on the isolated myocardium.

A Design for the Bioassay of Glycosides on Isolated Hypodynamic Myocardium.—The technical difficulties in preparing the papillary muscle for the measurement of the cardiotropic drugs are too great to make the method suitable for a routine assay. Its advantage is that it requires minute amounts of the drug, and for this reason it may find a place in the potency estimation of substances which are only available in small quantities, such as occurs in pilot experiments for purification of new glycosides.

The analysis of the available data has shown that the slope method and the threshold method both have the same order of precision. But the procedure of determining the threshold dose is by far less complicated than the alternating substitution of two drugs in the third stage. These methods require an adaptation of the dose scheme to the condition of the contraction of the muscle, which can only be observed during the experiment, and the exact data are only available when the photographic recording has been developed. Thus the method of choice is the threshold method, and the best design, as stated previously, is to prepare two papillary muscles from the same heart, and apply the unknown to one of them and the standard to the other. The uncertainty introduced by a different level of work can be reduced by starting the drug application on both muscles when they have reached approximately the same degree of contraction in comparison to the initial amplitude. The optimal starting point would be when the amplitude is about one-third of the initial contraction. The threshold dose for ouabain under these conditions would be about 2.5 μg. %, according to Equation 1.

SUMMARY

It is possible to compare isodynamic amounts of cardiac glycosides on hypodynamic myocardium.

The comparison can either take place on separate muscles at the stage when a threshold dose of the drug causes an increase in amplitude of the hypodynamic isolated heart muscle, *threshold method*, or the drugs can be alternatively substituted on the same muscle preparation at the stage of increasing amplitude, the *slope method*. The latter method is essentially a one-dose assay, and several experiments must be combined in order to determine the ratio of doses which exactly causes a continuously rising slope.

The relative potencies obtained by the two methods are in agreement within the error of the assay, showing that the eleven glycosides compared with ouabain as a standard can be mutually replaced at any level of work of the hypodynamic myocardium. The agreement of the potencies obtained by the isolated myocardium methods and the potencies obtained by the official cat assay method shows that all drugs have

the same margin of safety as ouabain. One exception may be Cerberin which according to our assay has a wider margin of safety than ouabain.

The threshold method seems most suitable for a bioassay and a design of the procedure is suggested.

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The Estrogenic and Uterine-Stimulating Activity of *Asclepias tuberosa*. A Preliminary Investigation*

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The fluidextract of *Asclepias tuberosa* was found to contain substances estrogenic to castrate rats. A method for separating these substances is proposed. The fluidextract was also found to exert an appreciable oxytocic-like action upon the uteri of several species of laboratory animals both *in vitro* and *in vivo*. The literature on *Asclepias tuberosa* is reviewed.

DURING the course of a pharmacological and chemical investigation of a proprietary formula used in the treatment of the symptoms associated with dysmenorrhea and the menopause, it was found that several of its ingredients, among which was *Asclepias tuberosa*, contained an estrogenic substance. This drug was also found to contain a principle exerting a comparatively powerful stimulating effect on the uteri *in vitro* and *in vivo* of various laboratory animals. The present report concerns a preliminary study of the estrogenic and uterine activity of this plant, and the chemical investigation conducted for the purpose of tracing the active principles.

HISTORY

Asclepias tuberosa, commonly known as pleurisy root or butterfly weed, is a member of the milkweed family, native to eastern North America, occurring in scattered dry fields from Ontario and Maine west to Minnesota, and south to Florida, Texas, and Chihuahua, and thence west to Arizona. The commercial drug consists of the dried root of *Asclepias tuberosa*, Linné, Fam. *Asclepiadaceae*, and is now mostly collected in Georgia and the Blue Ridge section of North Carolina. This is the only species of the genus that does not have a milky latex.

Although considerable work has been done on the poisonous species of this genus because of their widespread damage to livestock, relatively little work has been done on the medicinal species, *Asclepias tuberosa*. Of the three species recognized in the United States Pharmacopeia of 1840, this species is the only one used today to any extent. *Asclepias tuberosa* was last official in the N. F. V, but is still official in the Homeopathic Pharmacopeia.

The plant was a favorite remedy of Western and Canadian Indians who employed it medicinally as an expectorant, diaphoretic, and emetic, and as a food after thoroughly cooking to destroy the glucoside. A crude sugar was obtained by them from the flowers of the plant (1).

Millspaugh (2) claims that Schoepf introduced *Asclepias tuberosa* to the medical profession. It is evident that it soon became a favorite drug of the Eclectics as an expectorant and diaphoretic. It is mentioned by Beach (3) as early as 1843 as a remedy for pleurisy, as a sudorific, and as a carminative and antispasmodic in colic and flatulence. Asclepias is still a popular home remedy in the South for pleurisy. The Eclectics also employed the drug in the treatment of respiratory diseases and various uterine disorders (4, 5). The latter use was responsible for its appearance in several well-known proprietary remedies.

The rationale for the use of *Asclepias tuberosa* appears to have been purely empirical. Little or no pharmacology, in the modern sense, has been conducted on it. Felter (6) claimed that it slowed the action of the heart, lowered arterial tension, and relieved local hyperaemia by vasomotor control, as well as having a circulatory regulating action upon the sweat glands producing true diaphoresis. No experimental details were given, however.

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Chemical investigation of *Asclepias tuberosa* has been equally meager. In 1882, Clabaugh (7) separated from the drug a saponifiable fixed oil and an odorous, crystalline, sublimable stearoptin melting at 160° F. Quackenbush (8), in 1889, isolated from *Asclepias tuberosa* a crystalline glycoside believed to be identical with "Asclepiadin" previously isolated from *Asclepias cornuti*. In 1922, Neish and Burns (9), reported that the seed cake from *Asclepias tuberosa* was useless as a cattle food because of a poisonous glucoside present. The present investigators have separated considerable amounts of sucrose from the alcoholic extract of the commercial drug.

The drug used in this investigation was supplied by the St. Louis Commission Company and was authenticated pharmacognostically by Professor Heber W. Youngken of the Massachusetts College of Pharmacy, Boston. The test material represents portions from lots gathered during the years 1942 to 1948. As is usually the case with botanical drugs, a few marked differences in the potencies of certain lots were found. The over-all pharmacodynamic results, however, were of the same basic nature.

EXPERIMENTAL

Estrogenic activity.—The technique and procedures employed in determining the estrogenic activity of *Asclepias tuberosa* were essentially the same as those reported previously (10), wherein the estrogenic activity of the total formula and licorice, another ingredient, were determined. Insufficient time was available however, to conduct as detailed an investigation upon asclepias as upon licorice.

Initially, fluidextracts from several lots of drug, made by the U. S. P. method type process A using 38.5% ethanol and 60% isopropanol menstruums, were employed. Great variation in estrogenic activity was observed among these. Those demonstrating significant activity were estrogenic in doses between 0.45 and 1.76 cc. Others were less potent, several fluidextracts being found inactive when maximum volumes were injected. Generally, alcoholic Soxhlet extracts were found to be estrogenic in doses averaging 1 Gm. of whole drug.

The existence of estrogenic activity in the drug having been established, steps were taken toward the separation or concentration of the principle. The total phenol fraction of several of the fluidextracts and the alcoholic Soxhlet extracts was obtained by the modified Pincus procedure as described previously (10). Of these, the total phenols from a fluidextract made with 60% isopropanol appeared to be the most active. This fraction represented 0.56% of the whole drug. Complete estrus occurred in all ten of the test animals following injections of 56 mg. of this material. This work was conducted prior to our discovery that acid hydrolysis of licorice extract was unnecessary and that washing with saturated sodium carbonate solution as recommended by Pincus (11) resulted in a great loss of estrogenic content from licorice extracts.

A subsequent test on these washings from *Asclepias tuberosa* revealed that this treatment also removed a great deal of the estrogenic material from this drug. The extract obtained from the washings represented 0.188% of the whole drug. It proved to have essentially the same potency as the total phenols themselves, injections of 56 mg. producing a marked degree of estrus in all the test animals.

Pincus and others have shown that sodium carbonate solutions remove estriol quantitatively from mixtures of the three natural hormones. In view of this, the above findings indicate the possibility of the presence of estriol in this drug.

In order to insure the recovery of all the estrogen from the drug, a departure from the usual procedure was taken, wherein a fraction normally discarded was further processed. This fraction was the aqueous layer from the hydrolyzed extract after having been extracted with ether, which in this case was made alkaline with sodium hydroxide and again extracted with ether. The resultant ether extract was then extracted with dilute hydrochloric acid. The ether phase remaining was evaporated, yielding an extract representing 0.0719% of the whole drug. This was found, on bioassay, to be estrogenic in doses of 21.4 mg., somewhat greater in potency than the total phenols or the sodium carbonate washings.

The acid phase was neutralized with sodium hydroxide and extracted with ether. From this, on evaporation, an extract representing 0.0152% of the whole drug was obtained. Bioassay proved this fraction to be by far the most potent yet obtained from *Asclepias tuberosa*. Injections of 4.5 mg. put all the ten test animals in a complete state of estrus.

Because of the activity found in these latter two fractions, it is the opinion of these investigators that in addition to the possible presence of estriol, other estrogenic principles occur in *Asclepias tuberosa*. The fact that one of these active principles could be separated from alkaline solution with ether, and the other from ether with dilute hydrochloric acid certainly indicates that their chemical properties differ greatly from estriol.

Figure 1 illustrates the process involved in obtaining the various active fractions from *Asclepias tuberosa*.

Pharmacological Methods.—Experiments upon isolated tissue were conducted using the general methods described in previous papers (12–14). Experiments were made using isolated uterine tissue of the guinea pig, the rabbit, the rat, and in a few cases, of the human. The rat was found to be the animal of choice in *in vitro* experimentation since the results were usually more reproducible. The estrus cycle was also easily determined, a fact of marked importance in the success of any experiment. It was found that the uterine tissue of rats at anestrus responds much more uniformly than at other times of the cycle. The *in vivo* studies were based upon methods previously reported (12–14). Simultaneous tracings of uterine contractions, blood pressure, and usually respiration were made. The animals used in this work included the rabbit, the cat, and the dog. Drugs which were dealcoholized, buffered, and filtered are referred to hereafter as "prepared." *In vivo* doses are stated as total amount given.

Results In Vitro.—A group of ten experiments using uterine horns of the virgin guinea pig were made. In all cases concentrations of 1:10,000 of the prepared fluidextract of asclepias showed marked increases in activity accompanied by high tone and clonic contractions. In one case a concentration as small as 1:100,000 showed minor stimulation.

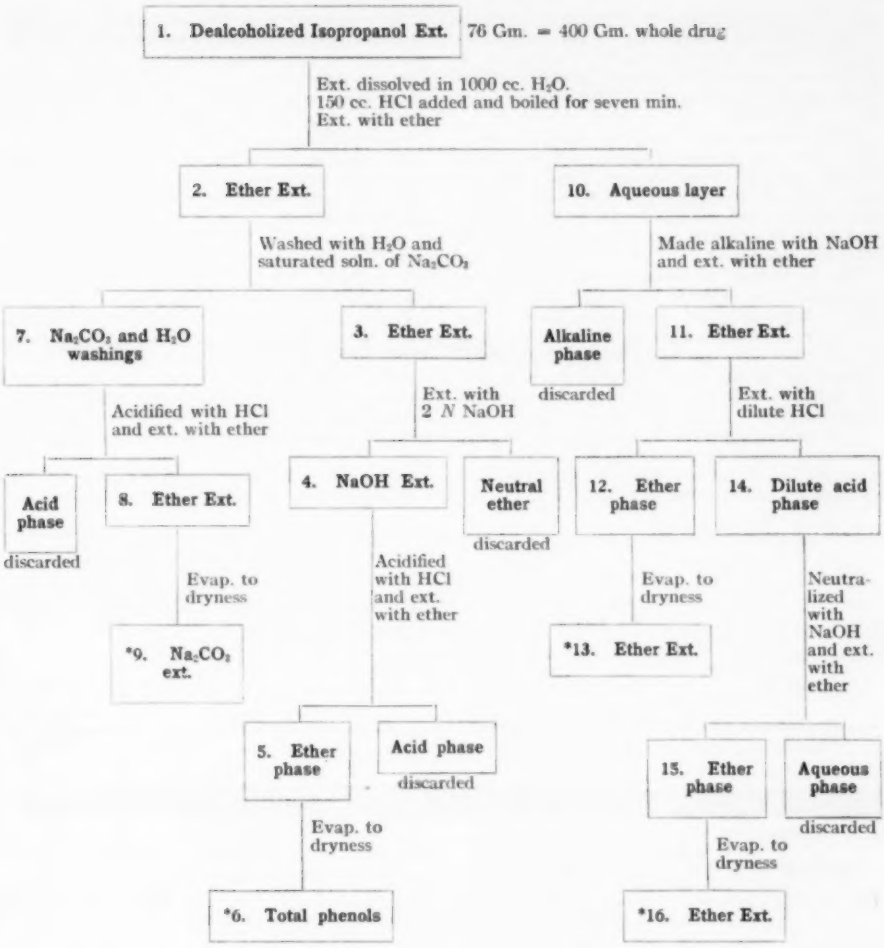

Fig. 1.—Separation of Estrogenic Fractions from *Asclepias tuberosa*.

* Active fractions.

When rabbit tissue was employed, the effective concentration of the fluidextract of asclepias was found to be 1:10,000. A water-soluble extract of asclepias also showed marked stimulation on rabbit tissue at a 1:10,000 concentration.

We were not able, however, to demonstrate the same degree of stimulation using uterine tissue of the rat as with the guinea pig and the rabbit. The highest concentration of the prepared fluidextract that demonstrated consistent stimulation was approximately 1:5000. A fraction which was separated by the method of Harnack (15) and believed to contain the glucoside "Asclepiadin" gave some stimulation with a 10 mg. concentration in a 100-cc. muscle chamber.

As a matter of interest, several experiments were made to compare the oxytocic effect of asclepias against the sedative effects of *Aletris farinosa* and *Piscidia erythrina*. A solution containing 50% each of the fluidextracts of asclepias and piscidia consistently showed the predominant sedative action of piscidia in a 1:5000 concentration. In comparing the action of asclepias against that of aletris, it was determined that a balance of effect was reached when rat uteri were exposed to a 1:500 concentration of a solution containing 1 part of asclepias to 149 parts of aletris.

In Vivo.—The *in vivo* studies were made using the various methods and modifications previously reported. Recordings of uterine contractions, blood

pressure, and respiration were taken simultaneously in the majority of experiments. A few intrauterine balloon tracings using the dog were completed. A total of 13 experiments using the dog, 17 experiments using the rabbit, and 36 experiments using the cat were made.

Of the 17 experiments using the rabbit, 16 showed stimulation of uterine activity, usually in very marked degree. Blood pressure usually showed some increase with irregularity. In general, respiration changes were not significant, despite the fact that in the chart shown (Fig. 2) apnea was observed after each injection.

The fluidextract of asclepias, usually prepared, was found to be potent in comparatively small dosage. Using the rabbit, intravenous doses as low as 0.01 cc. showed stimulation, but doses of 0.05 to 0.15 cc. were more commonly employed. Figure 2 shows a typical response. As dosage was increased, the stimulation likewise increased in almost quantitative degree.

In 6 experiments with the dog using our regular method of recording, stimulation was shown in all cases with doses of the prepared fluidextract of asclepias ranging from 0.01 to 0.3 cc.

There appears to be some substance in the fluidextract of asclepias which is somewhat toxic when administered intravenously to omnivorous species, since the higher doses sometimes resulted in death after a short period of time. This was not observed with the rabbit, which is herbivorous, nor on oral administration to rats. It was determined that death was due to respiratory arrest. We propose to further investigate this phenomenon.

Of the seven intrauterine balloon experiments attempted with the dog, four showed some increase in uterine activity. Figure 3 shows a typical result. Results of the three remaining experiments were negligible. A description and a discussion of this method appear in a previous report (14).

Experiments using the cat were divided into two series. One series of 27 experiments was made to determine the action of the prepared fluidextract of asclepias upon the uterus, blood pressure, and respiration, and another series, to date consisting of 9 experiments, was started to compare the action of asclepias against various oxytocics and uterine sedatives.

In the first series, stimulation of uterine activity was observed in 23 out of the 27 experiments.

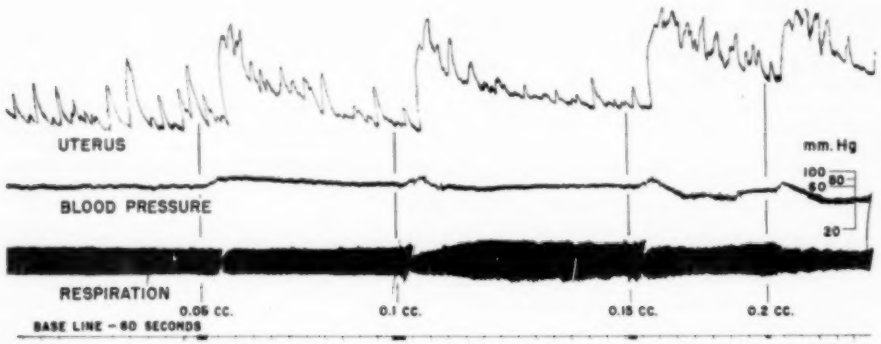

Fig. 2.—Effect on rabbit, 3.82 Kg. (parous), *in vivo*. Drug (intravenous): asclepias, fluidextract, dealcoholized, buffered, filtered, precipitated with lead subacetate.

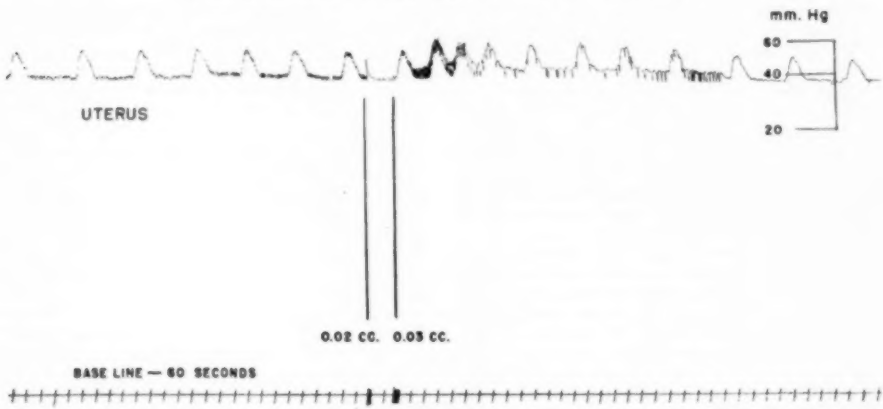

Fig. 3.—Effect on dog, 5.0 Kg., intrauterine balloon method. Drug (intravenous): asclepias, fluidextract, dealcoholized, buffered, filtered.

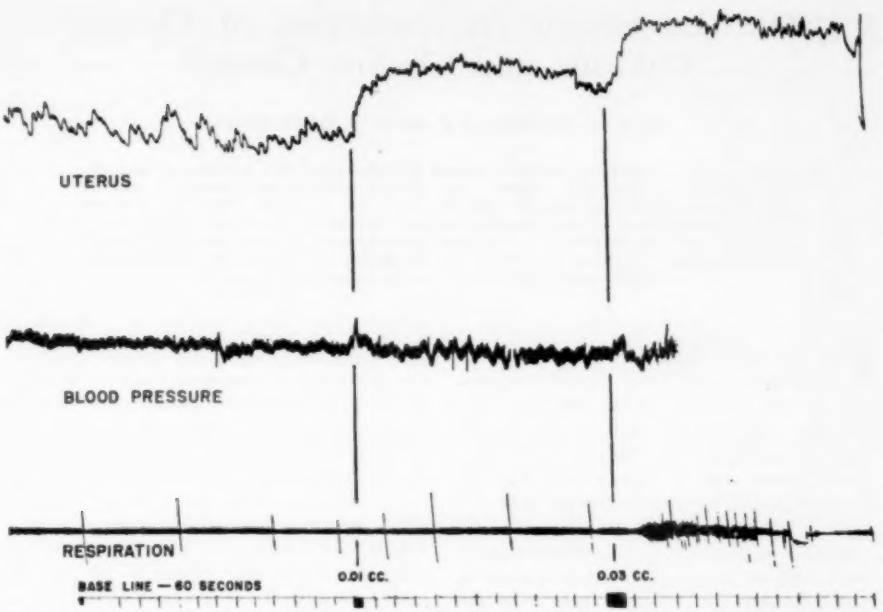

Fig. 4.—Effect on cat, 2.54 Kg., *in vivo*. Drug (intravenous): asclepias, fluidextract, dealcoholized, buffered, filtered.

Doses ranging from 0.01 to 1 cc. were employed. The average dose for marked stimulation was determined to be 0.05 cc. of the fluidextract of asclepias. In many cases, uterine contractions were of the clonic type with substantial rises in tonus. The drug was also found to stimulate intestinal tissue in the cat at approximately the same dosage. Figure 4, showing the effects of doses of 0.01 and 0.03 cc., respectively, of the prepared fluidextract of asclepias, is typical of the action usually demonstrated.

While the study of the comparative action of asclepias against various uterine acting drugs is incomplete, certain indications could be seen. It was found that 0.05 cc. of the prepared fluidextract of asclepias showed about the same oxytocic action as 0.1 unit of Pitocin.

A quantitative relationship was established in comparing the action of asclepias against aletris, a drug which in our hands was found to be a uterine sedative in certain species. It was shown that upon the administration of 1 cc. of a solution containing 0.005 cc. of the fluidextract of asclepias and 0.995 cc. of the fluidextract of aletris, the stimulating and sedative properties characterizing these two drugs were completely abolished. Solutions containing more than the above concentration of asclepias resulted in stimulation, while solutions containing less asclepias resulted in sedation.

SUMMARY

1. Preliminary investigation indicates that *Asclepias tuberosa* contains substances estrogenic to the castrate rat. The presence of estriol is suspected.

2. A method of chemical separation for these substances has been proposed.

3. The effect of the dealcoholized, buffered, and filtered fluidextract of *Asclepias tuberosa* upon the uterine tissue of various species of laboratory animals has been studied.

4. The drug was found to exert a definite stimulant action in small concentrations on uterine tissue both *in vitro* and *in vivo* in the great majority of experiments conducted.

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The Gravimetric Determination of Choline Chloride and Choline Citrate*

By R. E. PANKRATZ and F. J. BANDELIN

A gravimetric method for choline chloride and choline citrate utilizing ammonium reineckate as an analytical reagent. The choline salts are precipitated as choline reineckate in aqueous solution, filtered off, dried, and weighed. The data collected indicate that the method has a standard deviation of less than 0.2 per cent and that it is reliable and reproducible. It has the advantage over several other gravimetric methods in that it does not require the use of anhydrous solutions, nor does the precipitation need to be carried out in an anhydrous system.

THE ADVENT of choline salts as therapeutic agents has created a need for the determination of these salts in various pharmaceutical products. Although a number of analytical methods for choline have been reported, these generally leave something to be desired for the expedient and accurate determination of choline salts.

Choline is precipitated by a number of reagents and notably by the alkaloid precipitants such as the double salts with platinum, gold, mercuric, zinc, and cadmium chlorides (1–7), the iodine complexes by various iodine-potassium iodide solutions (8–12), by phosphotungstic acid (8, 13), by phosphomolybdic acid (8), by Mayer's reagent (14) (potassium mercuric iodide), by Dragendorff's reagent (14) (potassium bismuth iodide), and by ammonium reineckate (15–17).

Gakenheimer and Reguera (18) have reported a gravimetric method for choline chloride in pharmaceutical products which utilizes the precipitation of choline as the phosphotungstate. This method has the disadvantage of requiring an empirical factor to compensate for the solubility of choline phosphotungstate in absolute alcohol in which the precipitation is carried out. The use of absolute alcohol is also undesirable since liquid preparations of choline salts must be dried by heating under reduced pressure. Furthermore, the method claims an accuracy of ±4 per cent which, from the standpoint of analytical procedures, is inadequate for precise work.

More recently a gravimetric method for the determination of choline chloride and choline citrate has been described (19) in which the choline salts are precipitated in absolute alcohol as the double salt with cadmium chloride. This appears to be an accurate method with very slight deviation but it has the disadvantage of requiring an anhydrous system for precipitation.

A widely used method for choline determination is based upon the precipitation of choline as the reineckate and the subsequent colorimetric determination of an acetone solution of the isolated precipitate (20–23). The method has the disadvantage inherent in many colorimetric or spectrometric methods, namely that it lacks precision, since these methods have a range of variation of from 1 to 2 per cent. Although widely used as a colorimetric method for choline, the reineckate method has not been extensively or systematically investigated as a gravimetric method. With this end in view, the use of ammonium reineckate as an analytical reagent for the gravimetric determination of relatively pure choline salts was undertaken.

EXPERIMENTAL

Reagents:

Choline Dihydrogen Citrate.—The commercial product[1] recrystallized from a mixture of methyl and ethyl alcohols and dried overnight in a vacuum desiccator over magnesium perchlorate.

Choline Chloride.—Reference standard choline chloride obtained from the U. S. P. Revision Committee. This was dried at 110° and allowed to cool in a desiccator over magnesium perchlorate.

Ammonium Reineckate Solution.—Three grams of ammonium reineckate shaken with 100 ml. distilled water until saturated, then filtered. This solution should be prepared just before using.

Wash Solution.—Two milliliters of the saturated ammonium reineckate solution diluted with distilled water to 1 liter.

PROCEDURE

The choline chloride solution used is prepared by rapidly and carefully weighing 0.5 Gm. of the dried choline chloride and transferring it to a 200-ml. volumetric flask with the aid of distilled water. Two grams of citric acid are added and it is then diluted to volume with distilled water and mixed well. Pipette 15-ml. aliquots (containing 37.5 mg. of choline chloride) into 150-ml. beakers con-

* Received August 25, 1949, from the Control Laboratory of Flint, Eaton and Company, Decatur, Ill.

[1] Obtained from Calco Chemical Company, Bound Brook, N. J.

taining 25 ml. of distilled water. Carefully add 10 ml. of saturated solution of ammonium reineckate from a pipette, the end of which has been drawn to a small capillary, allowing it to run down the side of the beaker so that it forms a layer under the choline chloride solution without producing turbulence. The addition should be slow, requiring at least one minute. Mix by rotating the beaker gently and allow to stand for one hour, rotating from time to time.

Filter off the precipitates on tared 30-mm. low form sintered glass crucibles, using gentle suction. Rinse each beaker with three 15-ml. portions of wash solution and pass through the crucible containing the precipitate. The precipitate should not be allowed to draw completely dry during washing since the flat, platelike crystals form an almost impenetrable cake. After the last washing suck completely dry and place in oven at 100° for one hour. Cool in a desiccator and weigh as choline reineckate.

Choline dihydrogen citrate may be determined similarly by weighing 1.0 Gm. into a 200-ml. volumetric flask. Add 2.0 Gm. of citric acid and dilute to volume with water.

The precipitation, filtration, washing, and drying is carried out as given above, using the same precautions. Weigh as choline reineckate.

Calculations:

Weight of choline reineckate × 0.3304 = Grams of choline chloride

Weight of choline reineckate × 0.6989 = Grams of choline dihydrogen citrate

TABLE I.—THE GRAVIMETRIC DETERMINATION OF CHOLINE CHLORIDE AS CHOLINE REINECKATE

Precipitate, Gm.	Choline Chloride Found, Gm.	Choline Chloride Present, Gm.	Theory, %
0.1136	0.03753	0.03750	100.08
0.1137	0.03757	0.03750	100.19
0.1138	0.03759	0.03750	100.24
0.1137	0.03757	0.03750	100.19
0.1136	0.03753	0.03750	100.08
0.1138	0.03759	0.03750	100.24
0.1138	0.03759	0.03750	100.24
0.1136	0.03753	0.03750	100.08
0.1138	0.03759	0.03750	100.24
0.1138	0.03759	0.03750	100.24
0.1136	0.03753	0.03750	100.08
0.1136	0.03753	0.03750	100.08
0.0301	0.00995	0.0100	99.50
0.0302	0.00998	0.0100	99.80
0.0301	0.00995	0.0100	99.50
0.0604	0.01996	0.0200	99.80
0.0605	0.01999	0.0200	99.50
0.0604	0.01996	0.0200	99.80
0.0908	0.03003	0.0300	100.10
0.0908	0.03003	0.0300	100.10
0.0909	0.03003	0.0300	100.10
0.1210	0.03998	0.0400	99.95
0.1211	0.04001	0.0400	100.00
0.1209	0.03995	0.0400	99.87

Results of a number of determinations carried out at several levels of concentration using choline chloride are given in Table I.

Results of determinations on choline dihydrogen citrate are given in Table II.

TABLE II.—THE GRAVIMETRIC DETERMINATION OF CHOLINE DIHYDROGEN CITRATE AS CHOLINE REINECKATE

Precipitate, Gm.	Choline DHC Found, Gm.	Choline DHC Present, Gm.	Theory, %
0.1075	0.0751	0.0750	100.13
0.1070	0.0748	0.0750	99.73
0.1071	0.0749	0.0750	99.86
0.1073	0.0750	0.0750	100.00
0.1071	0.0749	0.0750	99.86
0.1073	0.0750	0.0750	100.00
0.1074	0.0751	0.0750	100.13
0.1070	0.0751	0.0750	100.13
0.1075	0.0751	0.0750	100.13
0.1075	0.0751	0.0750	100.13
0.1075	0.0751	0.0750	100.13
0.1075	0.0751	0.0750	100.13

DISCUSSION

The slow addition of the ammonium reineckate reagent is essential since it makes for the formation of large crystals of choline reineckate in the form of thin, flat, hexagonal plates. Rapid addition and excessive agitation causes the formation of a fine precipitate which filters with considerable difficulty. The use of an equivalent amount of hydrochloric acid in place of citric acid gives comparable results. The use of a very small quantity of ammonium reineckate in the wash water depresses the solubility of choline reineckate through its common ion effect (17) and has no detectable influence upon the weight of the precipitate.

The method yields consistent results with no apparent inherent error. Values obtained indicate a standard deviation of less than 0.1% for the chloride and less than 0.2% for the citrate.

The platelike structure of the crystals of choline reineckate offers an advantage in that no occlusion is likely. This is borne out by total sulfur analysis after Parr bomb decomposition which was 99.98% of theory. Water analysis by the Karl Fisher reagent indicates that the reineckate after drying is essentially anhydrous.

The method has the advantage of requiring no anhydrous solutions and that the precipitation need not be carried out in an anhydrous system. It is rapid and facilitates the determination of commonly encountered choline solutions without drying them.

The method has been applied in this laboratory to certain liquid pharmaceutical preparations and promises to be an extant method which may be extended to numerous choline products.

SUMMARY

Although a number of analytical methods for choline have been proposed, these methods lack either precision or expediency. The reineckate method which is generally employed as a colorimetric method for choline has the disadvantage of lack of precision inherent in colorimetric or spectrometric methods. The reineckate method has not been investigated as a gravimetric method for choline salts and such investigation reported

in this paper indicates this to be a reliable and sensitive method possessing advantages over methods now in use.

Under conditions of the procedure, choline chloride and choline citrate may be quantitatively precipitated from an acidified aqueous solution as choline reineckate in relatively coarse crystals. Structurally, these crystals are flat, hexagonal plates, which are readily washed and dried at 100°. The standard deviation calculated on results of determinations given in Tables I and II indicates less than 0.1 per cent deviation in the case of the chloride and less than 0.2 per cent in the case of the citrate.

The method requires no anhydrous solutions nor does the precipitation need to be carried out in an anhydrous system. It is rapid, accurate, and reproducible and may be applied to pharmaceutical products containing choline chloride or citrate and free from other substances precipitated by ammonium reineckate. It is especially useful for liquid products since these need not be dried as in methods requiring anhydrous precipitation.

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The Preparation of Seven N-Substituted Malonyl Guanidine Derivatives*,†

By NATHAN A. HALL‡ and LOUIS ARRIGONI§

Methods of synthesis are described for seven new N-substituted malonyl guanidine derivatives which are analogs of phenobarbital (a malonyl urea derivative).

IN THE search for new hypnotic drugs, the chemical relation between malonyl guanidine and barbituric acid (malonyl urea) has prompted considerable investigation, most of which has been fruitless. The only significant difference between the barbiturates and the malonyl guanidine derivatives is the configuration around the 2-position carbon atom in the nucleus. Several investigators (1–3) have studied 5-substituted malonyl guanidines pharmacologically but have observed no soporific properties. However, in 1942 Barré and Jacques (4) reported hypnotic action for one N-substituted malonyl guanidine, 2-phenyl imino 5,5-diethyl 1,3-dihydro 4,6-pyrimidinedione. This work suggested the synthesis of a number of similar compounds with varied N-substitution as possible central nervous system depressants.

A search of the literature reveals that a few N-substituted malonyl guanidines have been prepared by Höchster Farbwerke (5), Majima (6), Einhorn and von Diesbach (7), White (8), and Barré and Jacques (4). All of the compounds have had diethyl substituents on the position 5-carbon atom of the nucleus.

The compounds reported here have been prepared with 5-ethyl, 5-phenyl substitution; thus they are analogous to phenobarbital. The variation existed solely in the substitution on the imino nitrogen bonded to the carbon atom in position 2 of the nucleus for six compounds, and in the substitution on the nitrogen in position 1 for the other compound.

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The pharmacological investigation of the compounds of this series has been reported by Hall and Dille (9). No hypnotic action or other useful pharmacological property was observed.

EXPERIMENTAL

Preparation of 2-Methyl imino 5-ethyl 5-phenyl 1,3-dihydro 4,6-pyrimidinedione.—This compound was prepared by two different methods: (A) The sodium ethoxide condensation of methyl guanidine with a disubstituted malonic ester and (B) the treatment of a 5,5-substituted thiobarbiturate with methylamine.

Procedure A.—Seven and three-tenths grams of methyl guanidine base and 27 Gm. of ethyl phenyl ethyl malonate were heated for six hours under a reflux condenser with sodium ethoxide prepared by dissolving 5 Gm. of sodium in 150 cc. of absolute alcohol. The reaction mixture was concentrated almost to dryness under reduced pressure, the residue dissolved in water and precipitated by addition of 30% acetic acid until the mixture was acid to litmus. After standing overnight the precipitate was separated by filtration and recrystallized from large amounts of hot water. The yield was 10 Gm. of colorless, heavy, needle crystals melting at 218–219°[1] (41% of the theoretical).

Procedure B.—Five grams of 5-ethyl 5-phenyl thiobarbituric acid [prepared by the method of Tabern and Volwiler (10)] dissolved in 20 cc. of alcohol was added to 4 cc. of a 25% aqueous solution of methylamine in a pressure flask. Five grams of lead monoxide was added, and the flask was shaken vigorously. The mixture was then heated on a steam bath for ten hours with intermittent agitation. The precipitated lead sulfide was removed by filtration, washed 3 times with alcohol, and the combined washings and filtrate were evaporated to dryness on a steam bath. Purification by crystallization from hot water yielded 1.5 Gm. of a product identical with that produced by Procedure A above (31% of the theoretical).

Anal.—Calcd. for N, 17.13. Found: N, 17.08.[2]

Preparation of 2-Imino 5-ethyl 5-phenyl 1-methyl 3-hydro 4,6-pyrimidinedione.—Four and six-tenths grams of 2-imino 5-ethyl 5-phenyl 1,3-dihydro 4,6-pyrimidinedione [prepared by the method of Miller and Fischer (3)] was dissolved in 10 cc. of 2 *N* sodium hydroxide, and the solution was diluted to 60 cc. with alcohol. Five grams of methyl iodide and 0.5 Gm. of metallic copper were added and the mixture allowed to stand at room temperature for sixteen hours, then heated to 70° for four hours. The mixture was filtered, the filtrate evaporated to dryness, the residue extracted with chloroform, and the extract evaporated to dryness. The crude product was recrystallized from hot water and produced 1.5 Gm. of colorless, platelike crystals melting at 152–153° (48% of the theoretical).

Anal.—Calcd. for N, 17.13. Found: N, 17.28.

Preparation of 2-Isopropyl imino 5-ethyl 5-phenyl 1,3-dihydro 4,6-pyrimidinedione.—One and one-half grams of isopropylamine was mixed with a solution of 5 Gm. of 5-ethyl 5-phenyl thiobarbituric acid and 10 cc. of pyridine in ethyl alcohol. The reaction began immediately with the evolution of heat and hydrogen sulfide. The mixture was heated under a reflux condenser for eight hours until the evolution of hydrogen sulfide ceased. The pyridine catalyst was removed by steam distillation, and the water-insoluble solid mass remaining was recrystallized from 3:1 water-alcohol mixture. The yield was 3.2 Gm. of colorless, needle crystals melting at 184–185° (59% of the theoretical).

Anal.—Calcd. for N, 15.37. Found: N, 15.42.

Preparation of 2-*N* Butylimino 5-ethyl 5-phenyl 1,3-dihydro 4,6-pyrimidinedione.—This compound was prepared by the same method as the 2-isopropyl imino 5-ethyl 5-phenyl 1,3-dihydro 4,6-pyrimidinedione above. The product was recrystallized from a mixture of equal parts of water and alcohol. Colorless, light, needle crystals which melted at 155–156° were formed. The yield was 56% of the theoretical.

Anal.—Calcd. for N, 14.67. Found: N, 14.56.

Preparation of 2-Phenyl imino 5-ethyl 5-phenyl 1,3-dihydro 4,6-pyrimidinedione.—This compound was prepared by the same method as above except that the pyridine catalyst was omitted. The product was recrystallized from large quantities of alcohol. Colorless, fine, needle crystals which melted at 221–222° were obtained. The yield was 44% of the theoretical.

Anal.—Calcd. for N, 13.68. Found: N, 13.81.

Preparation of Ethylene bis-(2-imino 5-ethyl 5-phenyl 1,3-dihydro 4,6-pyrimidinedione).—This compound was prepared by the treatment of 10 Gm. of 5-ethyl 5-phenyl thiobarbituric acid with 1.5 Gm. of ethylenediamine and a pyridine catalyst by the method described above. The pyridine catalyst was removed by steam distillation, the mixture cooled and made acid to litmus with 10% acetic acid. The heavy, white amorphous precipitate was removed by filtration and crystallized from glacial acetic acid. The yield was 5 Gm. of colorless heavy, needle crystals melting with decomposition at 296–297° (51% of the theoretical).

Anal.—Calcd. for N, 17.21. Found: N, 17.05.

Preparation of 2-Para-amino phenyl imino 5-ethyl 5-phenyl 1,3-dihydro 4,6-pyrimidinedione.—This compound was prepared by the same method as outlined above with alcohol as the solvent and with a refluxing time of eighteen hours. The residue after steam distillation was dissolved in 0.5 *N* hydrochloric acid. The unreacted thiobarbituric acid was removed by filtration, and the product was precipitated by the addition of 0.5 *N* ammonium hydroxide until alkaline to litmus. The crude product crystallized from hot alcohol in tan, plate-like crystals which melted at 224°. The yield was 73% of the theoretical.

Anal.—Calcd. for N, 17.38. Found: N, 17.36.

Each compound prepared above was heated for one-half hour at 15 lb. pressure (120°) in an autoclave with 2 *N* hydrochloric acid. In every case, except with 2-imino 5-ethyl 5-phenyl 1-methyl 3-hydro 4,6-pyrimidinedione, phenobarbital was obtained (melting point 175°). The hydrolysis of 2-imino 5-ethyl 5-phenyl 1-methyl 3-hydro 4,6-pyrimidinedione produced N-methyl phenobarbital (Prominal) which was identified both by its melting point (176°) and nitrogen content.

[1] All melting points corrected.
[2] Nitrogen determinations were made by the micro Dumas method.

DISCUSSION

The isomeric compounds, 2-methyl imino 5-ethyl 5-phenyl 1,3-dihydro 4,6-pyrimidinedione, present an interesting problem in their synthesis. If the analogy between urea and guanidine is followed, it would seem likely that, when a substituted guanidine was used in a malonic ester condensation, the resulting product would carry a substituent group on the nitrogen atom in position 1 of the nucleus, since such a condensation product is formed with a substituted urea. However, in the case of methyl guanidine, malonic ester condensation yields a product with substitution on the imino nitrogen bonded to the carbon atom in the 2-position of the nucleus. Evidence of this phenomenon has been previously shown by Majima (6) and workers of Höchster Farbwerke (5). Further indication that the compound produced by base catalyzed substituted malonic ester condensation with a substituted guanidine carries an imino substituent is given by the preparation of the identical compound by treatment of the corresponding thiobarbiturate with methylamine.

SUMMARY

Seven new malonyl guanidine derivatives with nitrogen substitution have been prepared. The compounds are analogs of phenobarbital (a malonyl urea derivative). No hypnotic activity was observed for any member of this series.

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Note

A Note on the Preparation of Phenylmercuric Oleate*

By RICHARD H. BARRY,† ROBERT P. TANSEY, and LEWIS A. WALTER

PHENYLMERCURIC oleate has been used as a wood preservative (1–3), mildew inhibitor (4, 5), an agent for fungus-proofing gasketing material and glue-glycerol bonded corks (6, 7), and as a fungicide and bactericide in medicinal products. It is described in the chemical and patent literature as a viscous, oily material (8–12).

Our method of preparation results in the production of a pearly white, unctuous crystalline mass, which melts between 46.5° and 48°. The synthesis consists essentially of reacting a purified grade of oleic acid with phenylmercuric hydroxide under controlled temperature conditions. The compound formed is purified by crystallization from a suitable solvent.

EXPERIMENTAL

One hundred grams of phenylmercuric hydroxide[1] is slowly added with constant stirring to 100 Gm. of oleic acid (Emersol 233).[2] The temperature is maintained between 95° and 100° during the reaction period. The oily solution is cooled to 90° and approximately 350 ml. of isopropanol 99% is added to it. While the solution is still hot (80° to 85°), it is filtered by suction through a warm Büchner funnel into a warm filter flask. The filtrate is transferred to a beaker and is cooled in an ice-salt bath until crystallization is completed. The white precipitate is filtered off and drained and pressed dry by suction on a filter pad. The product is further air-dried until all the odor of the solvent has disappeared.

Purified by recrystallization from isopropanol 99%, the compound was obtained as a pearly white, unctuous crystalline mass melting at 46.5° to 48° (uncorr.). The material is stable when stored in a cool, dark place.

A chemical analysis[3] of two samples, using the U. S. P. XIII method for determining mercury in Mersalyl, gave an average result of 35.79% of mercury (theoretical 35.80%).

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* Received November 23, 1949, from the Research Laboratories of the Maltbie Chemical Company, Newark, N. J.
† Present address: Central Pharmacal Company, Seymour, Ind.
[1] Obtained from Edwal Laboratories, Inc., Chicago, Ill.
[2] Obtained from Emery Industries, Cincinnati, Ohio.

[3] The authors are indebted to Richard H. Robinson for the analytical data.

Book Reviews

Organic Syntheses. Vol. 29. CLIFF S. HAMILTON, Editor-in-Chief. John Wiley and Sons, New York, 1949. vi + 119 pp. 15 x 23.5 cm. Price $2.50.

This new volume of *Organic Syntheses* contains directions for the syntheses of thirty-four compounds. Many of them are of particular importance to pharmaceutical synthetic laboratories, such as the preparation of Raney nickel catalyst for use at low pressures, *tert* butylamine, *m*-aminobenzaldehyde dimethylacetal, *p*-nitrobenzonitrile, trimethylene oxide, α-phenylcinnamonitrile, and 2-thiophenealdehyde.

It is gratifying to note that the new volumes of *Organic Syntheses* include adequate cautions and describe the conditions under which adverse or dangerous reactions may occur so as to make the procedures much less hazardous. This seems to be particularly valuable since these procedures are commonly used in teaching laboratories, and are often assigned to technicians who may not be skilled enough to recognize the dangers involved. As usual, the syntheses in the new volume are accompanied by notes that are very useful to the operator and references to previous methods are included. A number of reaction types not previously covered in *Organic Syntheses* are contained in this new volume. The practice of having a cumulative index from Volume 20 is continued in this volume.

Practical Veterinary Pharmacology, Materia Medica and Therapeutics. 6th ed. By HOWARD J. MILKS. Alex Eger, Inc., Chicago, 1949. 720 pp. 15 x 23.5 cm. Price $10.

This book in its sixth edition retains much of the form and substance of former editions. While part of the book has been recently revised, it still retains much in form and content that needs revision. Approximately one-third of the present edition is devoted to pharmacy which may be permissible if the intent of the book is to serve the veterinary student in that field. If such is the case, a change in title to include pharmacy of pharmacognosy would be in order. The term "materia medica" is fast becoming obsolete. There is a loose use of scientific terms hardly justified in a text of this type. In some places confusion of thought and contradiction are evident.

Some phases of the subject are very well handled. Detailed study and experimental evidence on which conclusions are based would materially add to the value of the book.

Much extraneous material could be completely eliminated from the publication. Drugs and preparations no longer in use, and which haven't been in general use for years, should be considered as antiquated and could well be dropped entirely. Much of the material has evidently been drawn from the results of experimental evidence derived from human medicine. It is to be hoped that more experimental work could be done in the field of veterinary pharmacology where the pace is far behind research in human medicine.

Students in colleges of veterinary medicine using this work as a text in pharmacology should be encouraged to supplement their reading in pharmacology with assignments from the newer works coming out in pharmacology as applied to human medicine. This would give them a broader background in the subject backed by the latest experimental evidence. The relatively recent theory of stimulative medicine as compared to the old theory of repressive medicine, (illustrated by the old-time use of aconite, veratrum, antipyretics, etc.) might well be given greater emphasis for the present-day student. This book hardly handles this phase of the subject adequately.

This book is rather widely accepted and used in colleges of veterinary medicine as a text in this field. It is undoubtedly one of the better texts devoted to pharmacology in the field of veterinary medicine.

This book is well printed and bound and easily read. It is of good size for student use.—P. H. DIRSTINE.

The Essential Oils. Volume III. (*The Individual Essential Oils of the Plant Families Rutaceae and Labiatae.*) By ERNEST GUENTHER. D. Van Nostrand Company, Inc., New York, 1949. xvi + 777 pp. Illustrated. 15 x 23.5 cm. Price $10.

The third volume of this important series of books on the essential oils consists of monographs on the essential oils from members of the *Rutaceae* and *Labiatae* plant families. In each monograph the botanical and geographical origin, methods of cultivation of the plant from which the oil is derived, techniques of production and yield of oil, physical and chemical properties, chemical compositions, and use in the industry are clearly and concisely described and adequately covered. Succeeding volumes in the series will include comparable information on other essential oils until all have been covered.

Inorganic Chemistry in Pharmacy. By LLOYD M. PARKS, PAUL J. JANNKE, and LOYD E. HARRIS. J. B. Lippincott Co., Philadelphia, 1949. x + 298 pp. 18 x 26 cm. Price $6.

This book, a useful addition to the literature of pharmacy, is designed for students who have completed general and qualitative chemistry courses. The preparation, properties, tests, and uses of pharmaceutically and medicinally important chemicals are discussed in an understandable manner.

The material has been carefully organized. Chapter 1 is a brief introduction to the general factors underlying the action of drugs on cells. Chapter 2 is essentially a history of the development of the periodic table. Chapters 3 to 17 are arranged to

give a coherent discussion of the inorganic substances according to the periodic families. Within a particular family of elements, the material is divided into a general discussion of the elements, followed by an individual discussion of the compounds of a particular element in the order of hydrides of the element, metallic substitution products thereof, hydroxides (oxyacids and bases), and metallic substitution products thereof (salts). The history, natural occurrence, methods of formation and preparation, physical and chemical properties, and uses of each important substance are presented. Chapter 18 on isotopes, written especially by John E. Christian, is a readable and not too technical discussion of stable and radioactive isotopes of practical and theoretical utility in pharmacy and allied sciences. The bibliography will serve well for any reader interested in a more rigorous treatment of the subject.

A number of commendable features characterize this volume. The monographic treatment of individual chemicals and the liberal repetition of U. S. P. and N. F. passages have been consistently avoided. Complete bibliographies at the end of the chapters contain adequate references to the official compendia. Many references are recent, e.g., the danger of beryllium poisoning from cuts caused by broken fluorescent bulbs. Most welcome is the absence of complex diagrams of industrial equipment and processes.

Some features tend to detract from the evident merit of the book. Admittedly the authors presupposed a knowledge of chemistry on the part of the student; however, too great a part of the text is a tiring repetition of material previously covered in pharmacy history and chemistry courses. Parts of the text show an injudicious abuse of footnotes; over half of the printed matter on pages 20 and 21 consists of footnotes. The widely known Hubbard *Periodic Chart of the Atoms* loses many of its advantages in being reduced to a single page reproduction and could be best replaced by a simpler, more legible chart. Occasional inclusions of organic chemistry may be confusing to some students. A slight lack of uniformity is exemplified by "KO_4Cl" as compared to "K_2SO_4." More perplexing is the general equation on page 57, $M'X + M'$ (salt) $\rightarrow M'X + M'$ (salt).

A few statements in the text are open to doubt. It is stated in two equations that tin plus hot concentrated hydrochloric acid yields stannic chloride plus hydrogen, and that tin plus hot concentrated sulfuric acid yields stannous sulfate plus hydrogen. One is told that "the secondary alkali phosphates, M'_2HPO_4, are strongly hydrolyzed in solution and show an even greater alkaline reaction than the tertiary salts, M'_3PO_4."

Unfortunately, a number of typographical errors exist. The most serious is the electronic structural formula for sulfur dioxide in which six outer layer electrons are assigned to each oxygen atom, and eight to the sulfur atom. Most of the other errors comprise incorrect formulas ascribed to chromic sulfate, ferric subsulfate, cobaltous cyanide and colbaltic cyanide complex ions, and sodium uranyl cobalt acetate. Rutile is misspelled in both the text and index.

The authors have obviously covered their chosen subjects thoroughly. However, it is thought that some attention should have been given to the use of tantalum in surgery, helium in asthma, anesthesia and caisson disease, and lithium chloride in sodium chloride-free diets. The last item is certainly timely in view of recent evidence of possible dangers arising from excessive ingestion of lithium salts. Proposed structures for the ferric citrate complex might well have been included in the discussion of Ferric Citrochloride Tincture.

Like many of its predecessors of the Lippincott pharmacy series, this book has a very easy to read format on a good quality nonglare paper.

The volume has been designed as a text for undergraduate students and, as such, should prove very acceptable in an inorganic pharmaceutical chemistry course.—PIERRE F. SMITH.

WHO MAKES IT?

The National Registry of Rare Chemicals, Armour Research Foundation, 33rd, Federal and Dearborn Streets, Chicago, Ill., seeks information on sources of supply for the following chemicals:

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Atranorin
Physodic acid
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Canadine
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Ribose-1-phosphate
β-Ketostearic acid
β-Hydroxy palmitic acid
Adrenochrome
Pyruvamide
Rubrene
Anthrarobin
Azoisobutyrodinitrile
Laurone
3-Hexanethiol
Cyclohexanethiol
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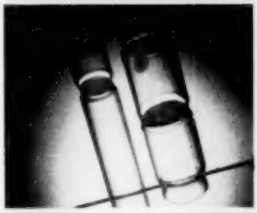
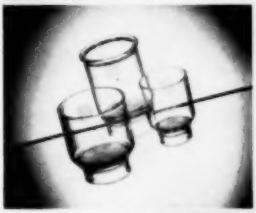
*For technical information regarding flow rates and pore sizes of Fritted Glassware, write Corning Glass Works for Bulletin B-80.





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